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COUNTERREGULATORY EFFECTS OF PTX3 ON INFLAMMATION
AND CELLULAR AGING

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

ANOVA	analysis of variance
APC	allophycocyanin
APMHR	age predicted maximal heart rate
BF%	body fat percentage
BIA	bioelectrical impedance
BMI	body mass index
BP	blood pressure
BSA	bovine serum albumin
CD	cluster of differentiation
CVD	cardiovascular disease
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein
GMFI	geometric mean fluorescent intensity
HR	heart rate
hTERT	human telomerase reverse transcriptase
IL	interleukin
IRF3	interferon-regulated factor-3
K_2EDTA	K_2 ethylenediaminetetraacetic acid
LPS	lipopolysaccharide

M1	pro-inflammatory macrophage
M2	anti-inflammatory macrophage
mAb	monoclonal antibody
MyD88	myeloid differentiation factor-88
NF-κB	nuclear factor kappa B
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PTX3	pentraxin 3
RER	respiratory exchange ratio
rh	recombinant human
rm	repeated measures
RPE	rating of perceived exertion
SASP	senescent-associated secretory phenotype
SAT	subcutaneous adipose tissue
SPSS	Statistical Package for the Social Sciences
T2DM	type 2 diabetes mellitus
TGF-β	transforming growth factor beta
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-alpha
TRIF	Toll/IL-1 domain-containing adaptor molecule
VAT	visceral adipose tissue
WB	whole blood

W:H	waist-to-hip ratio
VCO$_2$	carbon dioxide production
VO$_2$	oxygen consumption
VO$_{2max}$	cardiorespiratory fitness / maximal oxygen consumption

Abstract

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Pentraxin 3 (PTX3) is a vital regulator of innate immune function that has been shown to counterregulate pro-inflammatory signaling and protect against the development of cardiovascular disease (CVD). Less is known about how PTX3 may mitigate against CVD risk by regulating the pro-inflammatory response at the cellular level. Therefore, this dissertation details four manuscripts which aimed to examine the capacity of PTX3 to regulate the innate immune response of peripheral blood mononuclear cells (PBMCs) isolated from healthy adults. *Manuscript 1* examined the capacity of PTX3 to alter the inflammatory milieu following *in vitro* stimulation of isolated PBMCs with the pro-inflammatory lipid palmitate. In addition, *Manuscript 2* sought to examine how participation in acute exercise, a powerful anti-inflammatory behavior that reduces CVD risk, alters the inflammatory phenotype and response of mononuclear cells following *ex vivo* stimulation with lipopolysaccharide (LPS). *Manuscript 3* aimed to further elucidate the potential

impact of cardiorespiratory fitness on the capacity of PTX3 to stimulate an innate immune response prior to and immediately following acute exercise in aerobically trained and untrained individuals. Finally, *Manuscript 4* investigated the impact of healthy aging on plasma PTX3 concentrations and its relationship with telomere length in middle-aged compared to young adults. The capacity of isolated PBMCs to express a key cellular mechanism involved in maintaining longer telomere lengths, human telomerase reverse transcriptase (hTERT), following cellular stimulation with LPS, PTX3, and PTX3+LPS was also examined to address a mechanism that might explain how persistent exposure of circulating immune cells to the age-related pro-inflammatory milieu contributes to the shortening of telomere lengths.

CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death from non-communicable, preventable diseases in adults across the globe (Lozano et al., 2012). In the United States alone, over 40% of the adult population is expected to be diagnosed with CVD by 2030, accounting for a $1.1 trillion burden to the healthcare system (Heidenreich et al., 2011). These staggering statistics highlight the need to identify potential therapeutic targets that may attenuate the development and pathology of CVD with increased age.

Pentraxin 3 (PTX3) is an acute phase protein that was initially observed to be expressed by endothelial cells in response to stimulation with the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) (Lee et al., 1990). PTX3 has since been shown to be rapidly expressed by a variety of tissue and cell types following pro-inflammatory stimulation, including the lungs, ovaries, thymus, brain, skeletal and cardiac muscle, visceral and subcutaneous adipose tissue, vascular endothelial cells, fibroblasts, monocytes and monocyte-derived macrophages and dendritic cells, and maturing neutrophils (Alles et al., 1994; Lee et al., 1994; Introna et al., 1996; Polentarutti et al., 2000; Abderrahim-Ferkoune et al., 2003; Doni et al., 2006; Jallion et al., 2007; Osorio-Conles et al., 2011). In addition, PTX3 contains a 17-amino acid long leader peptide that is essential for its cellular secretion into circulation, and contributes to significant increases in plasma concentrations observed in response to systemic injection of the gram-negative endotoxin lipopolysaccharide (LPS) (Lee et al., 1993; 1994). This resultant increase in plasma PTX3 has contributed to its utilization as a biomarker of inflammatory perturbations linked to CVD and all-cause mortality (Rolph et al., 2002; Jenny et al. 2009).

Although PTX3 is commonly associated with acute inflammatory insult and disease pathology, Dias et al. (2001) utilized transgenic mice to show that the overexpression of PTX3 protects mice from death caused by systemic LPS injection. Furthermore, mice genetically

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modified not to express PTX3 present with increased systemic concentrations of pro-inflammatory cytokines, advanced progression of vascular inflammation and atherosclerotic plaque formation, and increased myocardial tissue damage following laboratory-induced heart damage (Salio et al., 2008; Norata et al., 2009). However, the reintroduction of exogenous recombinant human (rh)PTX3 in mice lacking the PTX3 gene is sufficient to reverse this phenotype, evidenced by lower systemic pro-inflammatory cytokine concentrations (i.e., IL-6) and reduced damage to cardiomyocytes (Salio et al., 2008). These findings suggest that elevated PTX3 concentrations observed during pro-inflammatory disease may be a compensatory response necessary to regulate an appropriate immune response during disease pathology.

At the cellular level, neutrophils are considered the primary cellular source of PTX3 (Jallion et al., 2007). More specifically, although mature peripheral neutrophils do not transcribe PTX3 in response to cellular activation with LPS, their precursors synthesize PTX3 throughout their maturation process and store ready-made concentrations that are released by mature neutrophils in response to cellular activation or following exposure to LPS and other pro-inflammatory stimulants, including TNF-α (Imamura et al., 2007; Jallion et al., 2007). In addition, Alles et al. (1994) have demonstrated that circulating monocytes are the only mature leukocyte to express and secrete PTX3 in response to direct cellular stimulation. Until recently, it was unknown how PTX3 may mediate the function of the innate immune system at the cellular level. However, Deban et al. (2011) has demonstrated that PTX3 binds to the leukocyte adhesion molecule, P-selectin, as a mechanism to prevent the excess transmigration of leukocytes into the vascular endothelium. Furthermore, PTX3 binds to and inhibits MD-2, a key molecule necessary for the activation of the LPS-mediated pro-inflammatory response through its interaction with toll-like receptor 4 (TLR4) on the surface of monocytes (Bozza et al., 2014). Likewise, exposure of

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circulating immune cells to elevated plasma PTX3 concentrations decreases cellular expression of key intracellular molecules involved in the pro-inflammatory signaling cascade within monocytes (Shiraki et al., 2016). As a result, PTX3 plays a vital role in regulating immune cell trafficking into inflamed or damaged tissue, and simultaneously downregulates the production of pro-inflammatory cytokines, including TNF-α and IL-1β, and the chemokine monocyte chemoattractant protein 1. In addition, PTX3 elicits the increased production of the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β) from neutrophil and macrophage cell lines (Bozza et al., 2006; 2014; Shiraki et al., 2016). These findings support the counterregulatory role of PTX3 in opposition to pro-inflammatory insult and suggest that additional investigations regarding the role of PTX3 as a potential mediator of CVD-related pro-inflammation are warranted. Therefore, *Manuscript 1* examined the capacity of PTX3 to alter the inflammatory milieu following *in vitro* stimulation of isolated peripheral blood mononuclear cells (PBMCs) with the pro-inflammatory lipid palmitate. Findings from this investigation suggest that physiologically relevant concentrations of PTX3 (500 pg/mL) mediate an anti-inflammatory response that may help prevent or counterregulate the development of CVD by enhancing immune cellular production of IL-10.

Evidence exists that supports physical activity as a powerful anti-inflammatory behavior that reduces the risk of age-related CVD pathology (Gleeson et al., 2011). In fact, Arem et al. (2015) has recently demonstrated that *any* participation in physical activity on a weekly basis can reduce early mortality by 20% and that engaging in at least 150 minutes of moderate-to-vigorous intensity physical activity suggested by the American College of Sports Medicine and Centers for Disease Control, procures a 31% reduction. Furthermore, plasma PTX3 concentrations have been shown to be significantly elevated in aerobically trained compared to untrained men (Miyaki et

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al., 2011), and in physically active adults who were previously inactive (Miyaki et al., 2012a). More importantly, elevated concentrations of plasma PTX3 in response to aerobic exercise training are associated with rapid and significant improvements in cardiovascular health (Miyaki et al., 2012b; Zempo-Miyaki et al., 2016). However, few studies have examined how physical activity and cardiorespiratory fitness influence the innate immune response of isolated monocytes following pro-inflammatory challenge, or how PTX3 may participate in this regulatory response. Interestingly, our laboratory has recently demonstrated that participation in an acute bout of submaximal exercise (75% of an individual's maximal oxygen consumption [VO_{2max}]) significantly increased plasma PTX3 concentrations for up to two hours during recovery from exercise (Huang et al., 2014; Slusher et al. 2015; 2016a). To the contrary, the PBMC production capacity of PTX3 following LPS stimulation was reduced and associated with the reduced production of IL-6 and IL-10 in healthy, normal-weight individuals who were physically inactive (Slusher et al., 2017a). Given that increased aerobic fitness, identified by an elevated VO_{2max}, has been closely linked to enhanced function of the immune system (Gleeson et al., 2011), *Manuscript 2* sought to examine how participation in acute exercise alters the inflammatory phenotype and response of mononuclear cells to *ex vivo* stimulation with LPS. Results from this investigation support our previous finding that acute exercise appears to elicit a pro-inflammatory phenotype of isolated monocytes, evidenced by the increased production of TNF-α and decreased production of IL-6 and IL-10 (Slusher et al., 2017a). As a follow-up, *Manuscript 3* sought to further elucidate the potential impact of aerobic fitness on the capacity of PTX3 to stimulate an innate immune response prior to and immediately following acute exercise. Results suggested that although elevated levels of aerobic fitness reduce the capacity of mononuclear cells to produce PTX3, the capacity of PTX3 to maintain the cellular production and release of IL-6 (a cytokine with known

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anti-inflammatory properties following exercise; Petersen and Pedersen, 2006) into the circulating microenvironment following acute exercise is enhanced with increased fitness.

Finally, aging is accompanied by chronic, low-grade elevations in systemic inflammatory cytokines, a phenomena known as *inflamm-aging* (Franceschi et al., 2000). While the sources of age-related inflammation are numerous, the accumulation of centrally located visceral adipose tissue occurring in the absence of weight gain or changes in body mass index (BMI) has been shown be a hallmark of age and a significant mediator of age-related pro-inflammatory profiles (Wu et al., 2007; Lumeng et al., 2011). As a result, elevated production and secretion of circulating pro-inflammatory cytokines contribute to the increased risk of premature morbidity and mortality from age-related diseases, including CVD and metabolic dysregulation (Kahn et al., 2006; Van Gaal et al., 2006). Telomere lengths are an indicator of cellular age (Benetos et al., 2001), and shorter telomere lengths are associated with increased concentrations of pro-inflammatory cytokines and increased morbidity and mortality from CVD (Cawthon et al., 2003; Epel et al., 2009; O'Donovan et al., 2011). Interestingly, elevated plasma PTX3 concentrations have recently been shown to be associated with longer telomere lengths in healthy, middle-aged adults, supporting the role of PTX3 as a biomarker of cellular aging. Therefore, *Manuscript 4* investigated the impact of healthy aging on plasma PTX3 concentrations and its relationship with telomere length in middle-aged compared to young adults. In addition, the capacity of isolated PBMCs to express a key cellular mechanism involved in maintaining longer telomere lengths, human telomerase reverse transcriptase (hTERT), following cellular stimulation with LPS, PTX3, and PTX3+LPS was examined to understand how persistent exposure of circulating immune cells to the age-related pro-inflammatory milieu may contribute to the shortening of telomere lengths.

CHAPTER 2

PENTRAXIN 3 IS AN ANTI-INFLAMMATORY PROTEIN ASSOCIATED WITH LIPID-INDUCED INTERLEUKIN 10 *IN VITRO*

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ABSTRACT

Pentraxin 3 (PTX3) is an acute phase protein expressed in response to pro-inflammatory stimuli during atherosclerosis. However, recent findings suggest that PTX3 is a counter-regulatory protein which enhances the anti-inflammatory response. **Objective:** Therefore, the capacity of PTX3 to alter the inflammatory milieu following *in vitro* stimulation of PBMCs with the pro-inflammatory lipid, palmitate, was examined. **Methods:** PBMCs from 17 healthy male donors were isolated and cultured under four separate conditions; 200 µmol/L palmitate, a physiologically relevant concentration of PTX3, in combination (pal + PTX3), and an unstimulated time-course control. **Results:** Palmitate-induced production of the counter-regulatory protein PTX3 was positively associated with the production of the anti-inflammatory cytokine interleukin 10 (IL-10) following *in vitro* stimulation of human PBMCs. Furthermore, stimulation of PBMCs *in vitro* with 500 pg/mL PTX3 elicited a significantly greater increase in IL-10 production compared to the palmitate stimulated conditions. However, PTX3 stimulation did not result in the production of the pro-inflammatory cytokines IL-1β, IL-6, and tumor necrosis factor alpha, and when combined with palmitate, did not alter the pro-inflammatory milieu from PBMCs in this study. **Conclusion:** These findings provide evidence supporting the role of PTX3 as a mediator of the anti-inflammatory response in physiologically relevant conditions, and suggests that PTX3 counter regulates the development of atherosclerosis by enhancing the production of IL-10.

INTRODUCTION

Atherosclerosis is a dynamic inflammatory condition involving the progressive accumulation of lipids and monocyte-derived macrophages within the vascular endothelium (Moore and Tabas, 2011). A number of studies demonstrate that the local expression of pro-inflammatory cytokines, including interleukin 1 beta (IL-1β), IL-6, and tumor necrosis alpha

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(TNF-α), exacerbate this pathogenic process (Libby et al., 2002; Kleemann et al., 2008). However, elevated expression of the anti-inflammatory cytokine IL-10 is also observed in concert with macrophage-derived pro-inflammatory cytokine production (Saraiva and O'Garra, 2010). IL-10 acts as a counter-regulatory protein that inhibits pro-inflammatory signaling and is athero-protective in mice (Thüsen et al., 2001; Han et al., 2010). Therefore, the identification of potential therapeutic agents that enhance the production of IL-10 may lead to treatment and prevention strategies which inhibit the atherosclerotic process.

Pentraxin 3 (PTX3) is an acute phase reactant expressed by circulating monocytes and resident macrophages in response to pro-inflammatory stimulation (Imamura et al., 2007; Doni et al., 2008). A recent study by Shiraki et al. (2016) demonstrated that concentrations of PTX3 (10 ng/mL) observed during pathological conditions downregulate the production of pro-inflammatory cytokines (IL-1β), and elicit the production of anti-inflammatory cytokines in response to pro-inflammatory stimulation of various macrophages cell lines. Unfortunately, although PTX3 is associated with the production of IL-10 in animal models (Dias et al., 2001; Bozza et al., 2006; 2014), Shiraki et al. (2016) did not investigate IL-10 or utilize *physiologically* relevant concentrations of circulating PTX3 on lipid-induced inflammation. The impact of PTX3 on IL-10 production in isolated human immune cells was also not examined.

Therefore, the purpose of this study was to examine the capacity of palmitate, an abundant nutritional 16-carbon chain saturated fatty acid commonly utilized as an *in vitro* model of lipid-induced inflammation, to increase PTX3 production following *in vitro* stimulation in peripheral blood mononuclear cells (PBMCs) from healthy male donors. In addition, the relationship of palmitate-induced PTX3 and the anti-inflammatory (IL-10) and pro-inflammatory cytokine (IL-1β, IL-6, and TNF-α) responses were assessed. It was hypothesized that the increased production

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of PTX3 following stimulation of PBMCs with palmitate would be positively associated with the production of anti-inflammatory and pro-inflammatory cytokines.

Furthermore, PBMCs were stimulated *in vitro* with physiologically relevant concentrations of circulating PTX3 typically observed in plasma under resting conditions to examine the anti-inflammatory impact of PTX3. It was hypothesized that PTX3 would elicit the production of IL-10, but not pro-inflammatory cytokines. Finally, PTX3 were added to PBMCs stimulated with palmitate to examine the hypothesis that PTX3 would augment palmitate-induced IL-10 production and attenuate the production of IL-1β, IL-6, and TNF-α *in vitro*.

METHODS

Subject Participation

Seventeen healthy male subjects were recruited to participate in the study. Subjects arrived at the laboratory at 6:00 am. Prior to participation, each subject provided informed consent and completed a medical history questionnaire. Subjects were excluded from participation if they had been previously diagnosed with inflammatory diseases/conditions (e.g., cardiovascular disease, chronic kidney or liver disease, diabetes) or under the current administration of medication known to alter inflammatory and/or metabolic profiles. Individuals who used tobacco products (cigarettes, cigars, chewing tobacco) or consumed an average of ten or more standard alcoholic beverages per week were also excluded. Finally, subject were instructed to undergo an overnight fast for at least eight hours and to abstain from alcohol, caffeine intake, and intense physical activity for at least 24 hours. The study was approved by the University Institutional Review Board.

Preparation of PTX3 and Palmitate Solutions

Recombinant human PTX3 (R&D Systems, Minneapolis, MN, USA) was reconstituted with phosphate buffered saline (PBS) (Fisher Scientific, Hampton, NH, USA) containing 0.1% fatty acid free bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA). A final concentration of 500 pg/mL was utilized for *in vitro* stimulation of PBMCs (described below). This concentration has been previously shown to be physiologically similar to circulating concentration of plasma PTX3 (Slusher et al., 2015), and was validated in our laboratory to be sufficient to stimulate the production of the anti-inflammatory cytokine IL-10.

Palmitate (Sigma Aldrich, St. Louis, MO, USA) was prepared according to Lee and colleagues (2012). In brief, palmitate was dissolved in 0.01M NaOH and solubilized at 70°C. Prepared palmitate was conjugated with fatty acid free BSA in PBS for 15 minutes at 50°C, resulting in a final molar ratio of 8:1. PBMCs were stimulated with a final concentration of 200 µmol/L, which has been previously shown to be sufficient to stimulate the production of pro-inflammatory cytokines (Shi et al., 2006).

Cell Culture for In Vitro PTX3 and Cytokine Production

A 10 mL whole blood sample was drawn from each subject's anticubital vein using a 21G butterfly needle into a tube containing K_2 ethylenediaminetetraacetic acid (K2EDTA) (BD Vacutainer, Franklin Lakes, NJ, USA). Whole blood samples were immediately centrifuged at 3000 RPM for 20 minutes at room temperature. The plasma supernatant was collected, centrifuged at 10,000×g for 10 minutes to obtain platelet free plasma, and immediately stored at −80 °C in cryogenic tubes in 500 µL aliquots for analysis of PTX3 using enzyme-linked immunosorbent assay according to manufacture instructions (R&D Systems, Minneapolis, MN).

The remaining buffy coat was collected and carefully layered over an equal volume of Ficoll-Paque (ρ = 1.077 g/mL; Sigma-Aldrich, St. Louis, MO, USA) in a conical tube and centrifuged at 400×g for 30 minutes at room temperature. The mononuclear cell layer was isolated, and washed with saline three times. Pelleted PBMCs were suspended in 1 mL RPMI 1640 media, counted manually by hemocytometer, adjusted to a final volume of 1.0×10^6 cells/mL, and cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin (Life Technologies, Carlsbad, CA, USA) in a 96-well cultured plate (Corning Incorporated, Corning, NY, USA). Plated PBMCs were stimulated with palmitate (200 µmol/L), PTX3 (500 pg/mL), or in combination (pal + PTX3) and incubated at 37°C for 24 hours. Unstimulated samples served as a time-course control. Culture supernatants were isolated and analyzed for PTX3, IL-1β, IL-6, IL-10, and TNF-α expression in duplicate by enzyme-linked immunosorbent assay methods according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical Analyses

Data analyses were performed using the Statistical Package for the Social Sciences (SPSS version 23.0). The effectiveness of palmitate stimulation on PTX3 production from PBMCs compared to time-course controls was assessed utilizing an independent *t*-test. A one-way ANOVA was conducted to compare the effects of each experimental culture condition (time-course control, palmitate, PTX3, pal + PTX3) on inflammatory cytokine release from PBMCs. Significant effects were further analyzed using Tukey's *post hoc* comparisons. To examine the relationships between PTX3 with pro- and anti-inflammatory cytokine production following palmitate stimulation, Pearson's correlation was utilized. Finally, the relationships between plasma

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PTX3 and the *in vitro* production of PTX3, pro- and anti-inflammatory cytokines under all stimulatory conditions were examined by Pearson's correlation. All data are presented as means $\pm$ S.E.M. unless otherwise stated with statistical significance being defined as a *P*-value ≤ 0.05.

RESULTS

Descriptive statistics, including resting plasma PTX3 concentrations, are presented in Table 1. Initial analysis revealed that palmitate elicited a significant increase in PTX3 compared to time-course controls [$t (7.882) = 32$, $p < 0.001$; Figure 1]. In addition, significant differences were observed among experimental culture conditions for IL-10 [$F_{(3, 64)} = 20.547$, $p < 0.001$], IL-1β [$F_{(3, 64)} = 31.296$, $p < 0.001$], IL-6 [$F_{(3, 64)} = 35.461$, $p < 0.001$], and TNF-α [$F_{(3, 64)} = 6.696$, $p = 0.001$; Figures 2A-D]. *Post hoc* analysis further revealed that palmitate, compared to time-course control conditions, elicited a significant increase in the anti-inflammatory cytokine IL-10 as well as the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. In addition, a positive association was observed between palmitate-induced PTX3 and palmitate-induced IL-10 (r = 0.538, $p = 0.026$), whereas no associations were observed between the production of PTX3 and IL-1β, IL-6, or TNF-α following palmitate stimulation (r = 0.394, $p = 0.118$; r = 0.357, $p = 0.159$; r = 0.012, $p = 0.965$, respectively; Figures 3A-D), even after controlling for plasma PTX3 concentrations.

Stimulation of PBMCs with 500 pg/mL PTX3 *in vitro* resulted in a significant increase in the anti-inflammatory cytokine IL-10 ($p < 0.001$) compared to time-course controls. However, PTX3 did not elicit an increase in the production of pro-inflammatory cytokines IL-1β, IL-6, or TNF-α. Statistical analyses further demonstrated that the production of IL-10 was greater following PTX3 compared to palmitate stimulation ($p = 0.003$; Figure 2A). Conversely, palmitate stimulation resulted in a greater production of the pro-inflammatory cytokines IL-1β and IL-6, and

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tended to elicit the greater production of TNF-α ($p = 0.157$) compared to PTX3 stimulation (see figure 2B-D). However, the palmitate-induced anti-inflammatory response (increased IL-10) was not augmented, and the pro-inflammatory response (increased IL-1β, IL-6, or TNF-α) was not attenuated by the physiological concentration of PTX3 utilized in this study. Finally, plasma PTX3 concentrations were not associated with the *in vitro* production of PTX3 or pro- and anti-inflammatory cytokines under any culture condition.

DISCUSSION

Results from this investigation demonstrate that lipid-induced production of the counter-regulatory protein PTX3 is positively associated with the production of the anti-inflammatory cytokine IL-10 following *in vitro* stimulation of human PBMCs. Furthermore, physiologically relevant concentrations of PTX3 typically observed in plasma under resting conditions (500 pg/mL) stimulate a significantly greater increase in IL-10 production from PBMCs *in vitro* compared to palmitate stimulated conditions. However, PTX3 stimulation does not elicit the production of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. These findings provide evidence to support the role of PTX3 as a mediator of the anti-inflammatory response, and suggest that PTX3 alters the inflammatory milieu associated with the development of atherosclerosis by enhancing IL-10 production.

This is the first study to demonstrate that palmitate elicits the production and release of PTX3 from human PBMCs *in vitro*. Circulating monocytes, which are the primary cell source responsible for PTX3 production by PBMCs (Maina et al., 2009), serve as a biological sensor reflective of tissue derived pathology (Bories et al., 2012). In addition, monocytes are widely considered to be the prominent cellular component of the lipid-induced vascular innate immune

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response upon differentiation into resident macrophages (Pols et al., 2011). Therefore, the positive association between palmitate-induce PTX3 and IL-10 production observed in this study suggests that the enhanced lipid burden within the vascular endothelium during atherosclerosis increases PTX3 and IL-10 production from resident monocyte-derived macrophages as a counter-regulatory response to mediate pro-inflammation.

Previous studies have also demonstrated that supra-physiological concentrations of PTX3 enhance the cellular production of IL-10 (Bozza et al., 2006; 2014; D'Angelo et al., 2009), whereas Shiraki et al. (2016) reported that stimulation of macrophages with pathological concentrations of PTX3 (10 ng/mL) inhibit pro-inflammatory signaling and cytokine production, supporting the notion that PTX3 is an anti-inflammatory protein. In corroboration of this posit, PTX3 increases the production of IL-10 without eliciting the production of pro-inflammatory cytokines when human PBMCs are stimulated *in vitro* with a physiologically relevant concentration of resting PTX3. These findings indicate two main hypotheses: i) increased resting concentrations of PTX3 may pre-dispose circulating monocytes toward an anti-inflammatory phenotype prior to their extravasation into the vascular endothelium, and ii) PTX3 production following inflammatory insult may stimulate a reciprocal anti-inflammatory response that therapeutically polarizes monocyte differentiation from an M1, pro-inflammatory, to an M2, anti-inflammatory, macrophage phenotype (Sun et al., 2015).

It is important to note that the addition of PTX3 did not alter the palmitate-induced pro-inflammatory milieu from PBMCs in this study. Mechanistic studies demonstrate that palmitate-induced pro-inflammatory signaling occurs through Toll-like receptor (TLR) 4 engagement and utilizes the myeloid differentiation factor-88 adaptor molecule to mediate activation of the nuclear factor kappa B transcription factor (TLR4-MyD88-NF-κB pathway) (Lee et al., 2003; Shi et al.,

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2006). This is in contrast to PTX3-induced anti-inflammatory signaling, which occurs through Toll-like receptor (TLR) 2, 3, and 4 engagement and depends on Toll/IL-1 domain-containing adaptor molecule-mediated activation of the interferon-regulated factor-3 transcription factor (TLR-TRIF-IRF3 pathway) (Bozza et al., 2006 2014). In addition, PTX3 induces Akt phosphorylation, which not only increases IL-10 production, but inhibits the TLR4-mediated pro-inflammatory signaling and regulates NF-κB transcriptional activity in mononuclear cells (Guha and Mackman, 2002; Saraiva and O'Garra, 2010; Shiraki et al., 2016). Compared to TRIF, TLR-mediated activation of the MyD88 adaptor protein is the more rapid and robust cellular pathway involved in inflammatory signaling (Kawai et al., 1999; Yamamoto et al., 2003). Therefore, the inability of PTX3 to alter the inflammatory milieu in this study, contrary to the study by Shiraki et al. (2016), may be due to the possibility that the dose of PTX3 that was utilized in this study was insufficient to phosphorylate Akt to a level required to augment palmitate-mediated IL-10 production or inhibit pro-inflammatory signaling, and suggests that additional research is warranted to determine the optimal physiological concentration of PTX3 necessary to counter pro-inflammatory signaling through Akt phosphorylation.

In conclusion, this investigation demonstrates that PTX3 production following *in vitro* stimulation of PBMCs with palmitate may serve as a counter-regulatory protein which preferentially facilitates an anti-inflammatory response characterized by elevated concentrations of IL-10. Thus, these findings provide additional evidence supporting the anti-inflammatory and athero-protective function of PTX3. Observations from this study are of particular interest for obese populations who present with decreased concentrations of plasma PTX3 and monocytes that are polarized towards an M1 phenotype (Yamasaki et al., 2009; Ogawa et al., 2010; Osorio-Conles et al., 2011; Bories et al., 2012; Chu et al., 2012; Miyaki al., 2013; Witasp et al., 2014; Slusher et

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al., 2015; 2016a), and further examination of the aforementioned responses in obese populations may provide additional insight into the clinical relevance of resting PTX3 concentrations. Consequently, additional studies are warranted to investigate the optimal *in vitro* and *in vivo* concentration of PTX3 necessary to correct inflammatory imbalances associated with PBMCs from individuals at risk for atherosclerosis and to elucidate the molecular targets specific to PTX3-induced IL-10 production. In addition, non-pharmaceutical intervention techniques which reduce the risk for inflammatory-associated diseases by enhancing resting and transient concentrations of circulating PTX3, such as acute and chronic aerobic exercise (Miyaki et al. 2011; Slusher et al., 2015), should be considered.

Table 1. Participant descriptive characteristics and resting plasma PTX3 concentrations

Variable	n = 17
Age (y)	24.53 ± 3.97
BMI	22.1 ± 1.78
Weight (kg)	71.02 ± 8.25
Height (m)	1.79 ± 0.08
Plasma PTX3 (pg/mL)	2366.08 ± 1853.47

Note: Data are presented as means ± SD. BMI = body mass index; PTX3 = pentraxin 3

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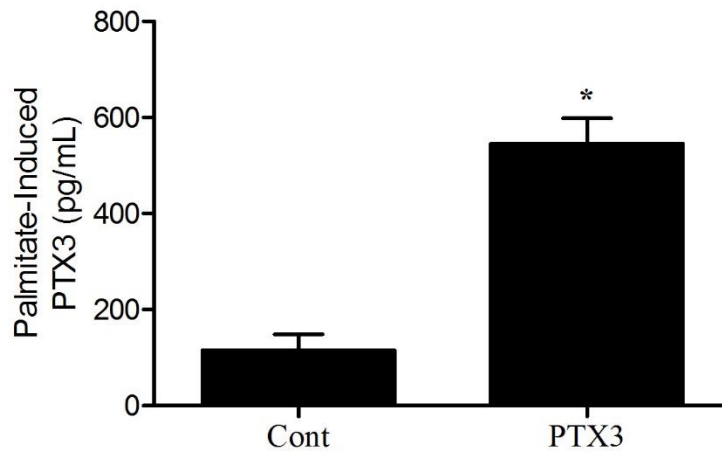
Figure 1. Stimulation of human PBMCs with palmitate *in vitro* induced a significantly greater production of PTX3 compared to the time-course control condition. The * indicates a significant difference between experimental culture conditions ($p < 0.050$). Data are presented as means ± SEM.
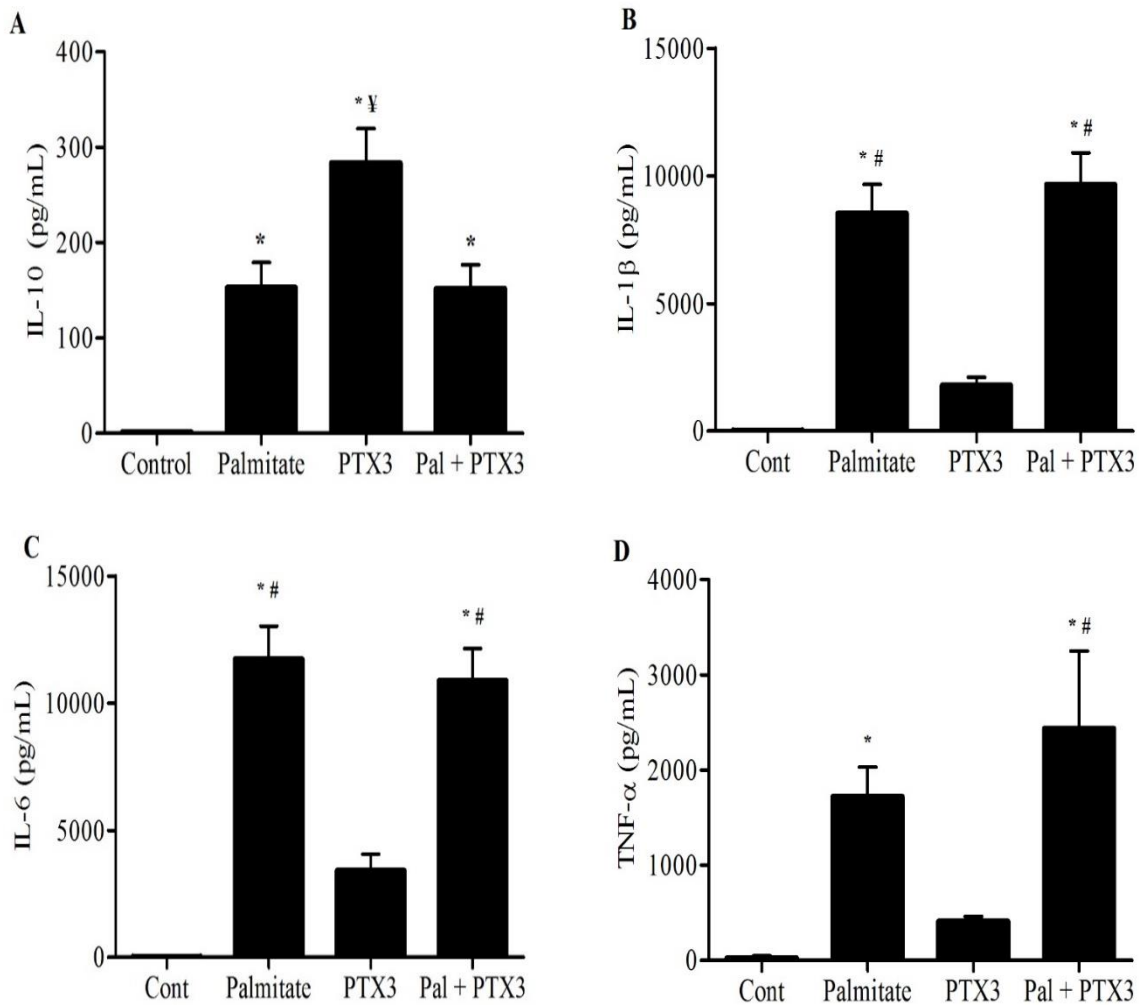
Figure 2. The production of anti-inflammatory (IL-10; panel A) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α; panel B-D) following the *in vitro* stimulation of PBMCs with palmitate, PTX3, and palmitate + PTX3 (Pal + PTX3). The * indicates a significantly greater production of inflammatory cytokines compared to time-course controls; the ¥ indicates a significantly greater production of the anti-inflammatory cytokine IL-10 compared to palmitate and Pal + PTX3; the # indicates a significantly greater production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α compared to PTX3; ($p < 0.050$). Data are presented as means ± SEM.

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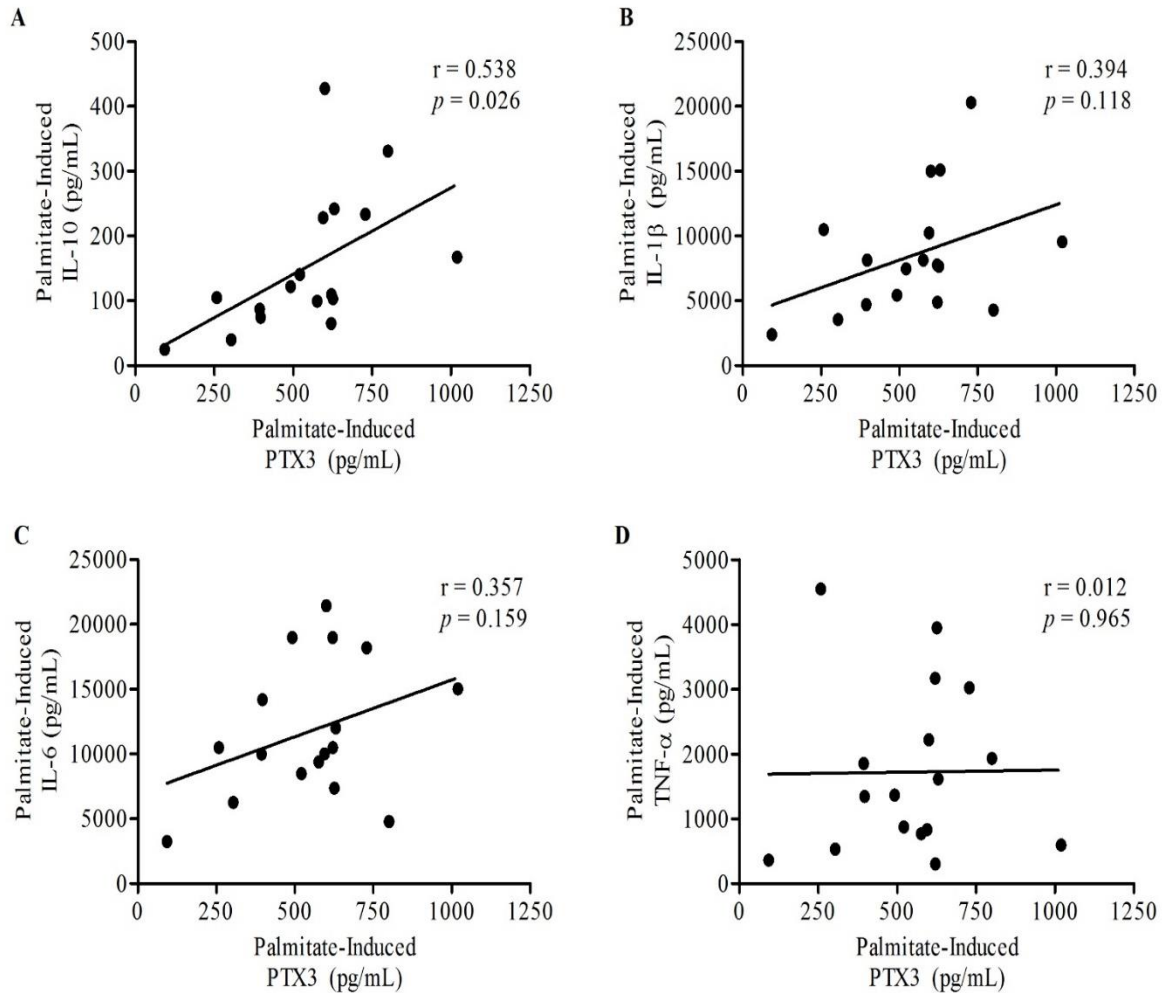
Figure 3. Associations of palmitate-induced PTX3 with palmitate-induced anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) following *in vitro* stimulation of PBMCs. A positive association was observed between palmitate-induced PTX3 and palmitate-induced IL-10 (panel A). However, no associations were observed between the production of PTX3 and IL-1β, IL-6, or TNF-α following palmitate stimulation (panel B-D), ($p \leq 0.050$).

CHAPTER 3

MAXIMAL EXERCISE ALTERS THE INFLAMMATORY PHENOTYPE AND RESPONSE
OF MONONUCLEAR CELLS

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ABSTRACT

Purpose: Monocytes express the CD14 receptor that facilitates lipopolysaccharide (LPS) ligation to toll-like receptor 4 (TLR4) to elicit production of interleukin (IL)-6, IL-10, and tumor necrosis factor alpha (TNF-α). However, pro-inflammatory conditions, such as strenuous exercise, increase the percentage of monocytes expressing CD16, a receptor that enhances LPS stimulated TNF-α production. Therefore, we examined whether maximal treadmill exercise would alter the inflammatory phenotype of *classical* (CD14$^+$/CD16$^-$) and *pro-inflammatory* monocytes (*intermediate* [CD14^{++}/CD16$^+$] and *non-classical* [CD14$^+$/CD16^{++}]), evidenced by changes in TLR4, CD14, and CD16 receptor expression, and their inflammatory response to *ex vivo* LPS stimulation. **Methods:** Human mononuclear cells from 25 male participants (age: 24.2 ± 4.0 yr.) were isolated prior to and following exercise to assess TLR4, CD14, and CD16 expression by flow cytometry and *ex vivo* production of LPS-stimulated inflammatory cytokines (IL-6, IL-10, and TNF-α). **Results:** Exercise reduced the percentage of *classical* monocytes and increased the percentage of *intermediate* and *non-classical* monocytes. In addition, TLR4 expression decreased on *classical* and *intermediate* monocytes, but not the *non-classical* monocyte subset. Furthermore, while CD14 expression decreased on all monocyte subsets, CD16 expression increased on *intermediate* monocytes only. In parallel with these phenotypic changes, the inflammatory milieu shifted towards a pro-inflammatory response following LPS stimulation (decreased IL-6 and IL-10 and increased IL-6 to IL-10 ratio and TNF-α production). **Conclusion:** These findings demonstrate that acute maximal exercise elicits a pro-inflammatory phenotype of isolated monocytes exposed to LPS and highlight potential mechanisms that will help elucidate the role of acute and chronic exercise on the innate immune response of circulating monocytes.

INTRODUCTION

Monocytes help initiate the innate immune response necessary to protect the host from "foreign" pathogens. Among peripheral blood mononuclear cells (PBMCs), monocytes are the primary cell type that express toll-like receptor 4 (TLR4), a transmembrane, pattern recognition receptor that stimulates the rapid production of inflammatory cytokines (interleukin-6 [IL-6], IL-10 and tumor necrosis factor alpha [TNF-α]) in response to the Gram-negative lipopolysaccharide endotoxin (LPS) (Pålsson-McDermott and O'Neil, 2004; Vaure and Liu, 2014). This activation occurs via CD14, a cell surface receptor on monocytes that facilitates LPS ligation to TLR4 and the subsequent inflammatory response (Wright et al., 1990; Kim and Kim, 2014). However, monocytes are a heterogeneous population that are subdivided based on the co-expression of the CD14 and CD16 receptors (Passlick et al., 1989). *Classical* monocytes express high levels of the CD14 receptor, but no CD16 (CD14$^{+bright}$/CD16$^-$), and produce IL-6 (a pro- *and* anti-inflammatory cytokine) and the anti-inflammatory cytokine IL-10 following LPS stimulation (Franenberger et al., 1996; Petersen and Pedersen, 2006; Shalova et al., 2012). To the contrary, *pro-inflammatory* monocytes express both CD14 and CD16 (CD14$^+$/CD16$^+$), and are further categorized as *intermediate* (CD14$^{+bright}$/CD16^{+dim}) and *non-classical* (CD14^{+dim}/CD16$^{+bright}$) based upon CD14 and CD16 expression patterns. In addition, *intermediate* and *non-classical* monocytes express elevated levels of TLR4 relative to *classical* monocytes (Simpson et al., 2009), and upon LPS stimulation, *intermediate* monocytes produce IL-6, whereas the CD16 receptor interacts with LPS ligation to TLR4 and enhances production of the pro-inflammatory cytokine TNF-α (Belge et al., 2002; Pinheiro da Silva et al., 2007; 2008; Cros et al., 2012). Given that the inflammatory phenotype of circulating monocytes predicts their innate immune function upon entry into tissue as macrophages (Bories et al., 2012), interest in monocyte heterogeneity and the potential impact

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of CD14 and CD16 receptor expression patterns on the LPS-induced inflammatory response has received increased attention in the literature.

The perturbation of acute exercise provides a model for altering receptor expression patterns on monocyte subsets, and for examination of the subsequent concomitant immune response to LPS stimulation. For example, acute bouts of submaximal aerobic exercise increase the percentage of *pro-inflammatory* monocytes in circulation (Wong et al., 2011; Radom-Aizik et al., 2014). Additionally, while *classical* monocytes express reduced levels of TLR4 expression during recovery from exercise, TLR4 surface expression has been shown to increase on both *intermediate* and *non-classical* monocytes (Hong and Mills, 2008; Simpson et al., 2009; Booth et al., 2010). Furthermore, Radom-Aizik et al. (2014) have revealed that monocytes demonstrate elevated expression of numerous pro-inflammatory genes following acute intense cycling (82% VO_{2peak}). Although these findings suggest that acute exercise may also elicit a pro-inflammatory phenotype of monocytes, none of the aforementioned studies examined the impact of acute exercise on CD14 or CD16 receptor expression on each monocyte subset, which may provide additional insight into their responsiveness to *ex vivo* stimulation with LPS. Similarly, while recent studies have shown that acute exercise decreases the capacity of cultured whole blood samples and individual monocytes to produce inflammatory cytokines following LPS stimulation (Dimitrov et al., 2017; Durrer et al., 2017), no studies have stimulated isolated PBMCs with LPS. This methodological approach removes the inflammatory contribution of neutrophils that express TLR4 and produce inflammatory cytokines following LPS stimulation of whole blood samples (Dubravec et al., 1990), and may provide a more effective investigation of the *cumulative* inflammatory status of monocytes exposed to elevated concentrations of LPS following exercise (Selkirk et al., 2008). Therefore, the aim of this study was to examine the impact of acute exercise

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on the inflammatory phenotype of a standardized concentration of PBMCs and their responsiveness to *ex vivo* LPS stimulation. It was hypothesized that the decreased percentage of *classical* monocytes expressing lower levels of TLR4 following acute exercise would accompany the reduced expression of CD14. In addition, the mobilization of *pro-inflammatory* monocytes expressing elevated levels of TLR4 following acute exercise was hypothesized to accompany the increased expression of CD14 and CD16. Finally, changes in the distribution of monocytes and their phenotypic characteristics were hypothesized to shift the LPS-stimulated inflammatory response towards a pro-inflammatory milieu, evidenced by the decreased production of IL-6 and IL-10, and the elevated production of TNF-α following *ex vivo* stimulation with LPS. The ratio of IL-6 to IL-10 was also examined prior to and immediately following exercise as an indicator of a pro-inflammatory response (Taniguchi et al., 1999).

MATERIALS AND METHODS

Research participants

A total of twenty-five healthy male research participants (age 24.2 ± 4.0 yr.; body mass index 21.96 ± 1.86 kg/m^2; mean $\pm$ SD) were recruited and completed the study. Twelve subjects were classified as aerobically trained (≥ 150 minutes of moderate to vigorous *aerobic* exercise per week) and 13 subjects were classified as aerobically untrained (< 150 minutes of any moderate to vigorous physical activity per week). Of note, female participants were excluded due to the potential influence of gender on the LPS-stimulated inflammatory response following intense exercise (Abbasi et al., 2013). Prior to participation, each research participant provided informed consent and completed a medical history questionnaire and 7-day physical activity recall to verify physical activity levels. Research participants were absent of any preexisting inflammatory

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disease(s) and were not currently under the administration of medication known to alter inflammatory profiles. In addition, research participants were nonusers of tobacco products (cigarettes, cigars, chewing tobacco) and consumed ten or less standard alcoholic beverages per week on average. The University's Institutional Review Board approved the study.

Exercise testing procedure

Research participants arrived at the laboratory at 6:00 o'clock in the morning following an 8-hour overnight fast and a 24-hour period without alcohol, caffeine intake, and intense physical activity. The testing session began with the familiarization with all instruments and procedures, followed by an assessment of height and weight utilizing standard medical equipment. Finally, an assessment of maximal oxygen consumption (VO_{2max}) was performed, utilizing a treadmill test administered in gradation (absolute VO_{2max} 3.75 ± 0.79 L $O_2 \cdot min^{-1}$; relative VO_{2max} 53.77 ± 10.34 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$; time to completion 798.24 ± 167.27 seconds; mean $\pm$ SD). The administered VO_{2max} test was performed according to previously published methods (Slusher et al., 2017a). In brief, the exercise protocol began with a 3-minute warm-up period at 4.83 kilometers per hour with 0% grade followed by 2-minute exercise stages. Speed was increased during the first 2-minute stage to elicit $80\% \pm 5$ beats per minute of the research participant's age predicted maximal heart rate (APMHR; 220-age), and allowed to reach steady-state during the second 2-minutes. Upon completion of stage two, grade was increased 2% with each subsequent 2-minute stage, while speed remained constant until the research participant reached voluntary exhaustion. VO_{2max} was determined using ParvoMedics Metabolic Measurement System (ParvoMedics, Sandy, UT, USA). Heart rate (HR) and rating of perceived exertion (RPE) were assessed by HR monitors (Polar T31, Polar Electro, Kempele, Finland) and the Borg RPE scale, respectively, and recorded during the

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final 15 seconds of every exercise stage. Rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were also assessed and averaged every 15 seconds to calculate respiratory exchange ratio (RER: VCO_2/VO_2). Criteria for attaining VO_{2max} included a plateau in O_2 consumption (defined as a failure to increase oxygen uptake by 150 mL/min with increased workload) or three of the following: RER $\geq$ 1.1, HR within 10 bpm of research participant's APMHR, an RPE $\geq$ 19, and a blood lactate $\geq$ 8.0 mmol/L (assessed by finger prick; LactateScout+; EKF Diagnostics; Cardiff, Wales).

Peripheral blood mononuclear cell isolation

A whole blood sample (10 mL) was drawn from each subject's antecubital vein prior to and immediately upon completion of exercise using a 21G butterfly needle into a tube containing K_2 ethylenediaminetetraacetic acid (K_2EDTA) (BD Vacutainer, Franklin Lakes, NJ). Whole blood samples collected prior to maximal exercise were kept at 4°C until the whole blood samples were collected immediately following acute maximal exercise, then simultaneously centrifuged at 3000 rpm for 20 minutes at room temperature. The leukocyte buffy coat was collected and carefully layered over an equal volume of Ficoll-Paque (ρ = 1.077 g/mL; Sigma-Aldrich, St. Louis, MO, USA) in a conical tube and PBMC were further isolated by centrifugation at 400×g for 30 minutes at room temperature. Isolated PBMCs were saline washed three times, suspended in 1 mL RPMI 1640 media, counted manually by hemocytometer, and adjusted to a final volume of 1.0×10^6 cells/mL.

Flow cytometry

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Aliquots of 1.0×10^6 cells/mL were washed, and pelleted cells were suspended in 100 µL of phosphate-buffered saline (PBS) and incubated with 5 µL of Fc receptor blocking solution (Biolegend, San Diego, CA, USA) for 10 minutes at room temperature to prevent non-specific binding. Cells were labelled with either 2.5 µL of an fluorescein (FITC) conjugated anti-CD14 (IgG1, clone: HCD14; Biolegend), phycoerythrin (PE) conjugated anti-TLR4 (IgG2a, clone: HTA125; Biolegend), and allophycocyanin (APC) conjugated anti-CD16 (IgG1, clone: 3G8; Biolegend) monoclonal antibody (mAb) or 5 µL of an appropriate isotype control. The optimized concentrations were determined prior to the study. Labelled cells incubated for 45-minute in the dark at 4°C then fixed with 500 µL of 1% paraformaldehyde (Biolegend) for 30 minutes in the dark at room temperature. Fixed cells were washed, suspended in 500 µL of PBS, and stored in the dark at 4°C prior to analysis on a FACSCalibur flow cytometer within 24 hours using CELLQuest Pro software (BD DBiosciences, San Jose, CA, USA).

Forward and 90° side scatter established primary gates for monocytes (Figure 1A), and secondary gates were established against FITC and APC fluorescence to identify monocyte subtypes based on CD14 and CD16 receptor expression patterns according to Mukherjee et al. (2015) (Figure 1B). TLR4, CD14, and CD16 density was assessed as geometric mean fluorescent intensity (GMFI) on $CD14^+$, $CD14^+/CD16^+$ monocyte populations as well as on *classical* ($CD14^{+bright}/CD16^-$), *intermediate* ($CD14^{+bright}/CD16^{+dim}$), and *non-classical* ($CD14^{+dim}/CD16^{+bright}$) monocytes subsets. Negative gates were established using the appropriate isotype controls to account for non-specific binding of Ig and to set the voltages for each fluorescence detector filter. To exclude overlapping emission spectra and control for electronic color compensation, the fluorescence of each conjugated mAb were analyzed in isolation using one-color analysis.

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Assessment of LPS-induced inflammatory cytokines

Aliquots of 1.0×10^6 cells/mL were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum, 1% penicillin, and 1% streptomycin (Life Technologies, Carlsbad, CA, USA) in a 96-well cultured plate (Corning Incorporated, Corning, NY, USA). Plated PBMCs were stimulated with LPS (10 ng/mL) and incubated at 37°C with 5% CO_2 for 24 hours as previously indicated (Slusher et al., 2017a). Unstimulated samples served as a time-course control. Culture supernatants were isolated and analyzed for IL-6, IL-10, and TNF-α in duplicate by enzyme-linked immunosorbent assay methods according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analyses

The Statistical Package for the Social Sciences (SPSS version 24.0) performed all data analysis. Notably, no differences in the monocyte phenotype or inflammatory response were observed among trained and untrained subjects. Therefore, subsequent analyses were performed in all subjects as a whole. Initial tests were performed to determine the presence of statistical outliers using interquartile ranges, and values that fell outside the lower and upper limits were objectively removed from analysis (Durrer et al., 2017). Assumptions of normality were examined using a Shapiro-Wilk test, and non-normal data ($p \leq 0.05$) were log transformed to reduce skewness and kurtosis. Welch's analysis of variance (ANOVA) examined differences in the distribution of monocyte subsets, and TLR4 and CD14 expression patterns prior to exercise among *classical, intermediate* and *non-classical* monocyte subsets, with significant differences assessed with Games-Howell's *post hoc* comparisons. Differences in CD16 expression patterns between *intermediate* and *non-classical* monocytes were also examined using an independent *t*-test prior to

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exercise. Paired *t*-tests examined the impact of acute exercise on the percentage and the TLR4, CD14, and CD16 expression density on *classical, intermediate* and *non-classical* monocyte subsets. To examine differences in monocyte subset responses to acute exercise, Welch's ANOVA compared differences in the percent changes (pre to post exercise) in monocyte subset distributions, and TLR4 and CD14 receptor expression patterns among *classical, intermediate,* and *non-classical* monocyte subsets, with significant differences assessed with Games-Howell's *post hoc* comparisons. Likewise, an independent *t*-test compared differences in the percent change in CD16 receptor expression between *intermediate* and *non-classical* monocyte subsets. In addition, paired *t*-tests examined the impact of acute exercise on the *ex vivo* production of IL-10, IL-6, and TNF-α following LPS stimulation. Finally, Pearson's correlations were utilized to examine the relationships between cardiorespiratory fitness (VO_{2max}), the percent changes in the phenotypic characteristics of monocytes (subset distributions and TLR4, CD14, and CD16 receptor expression), and their *ex vivo* production of inflammatory cytokines following acute exercise. The Benjamini-Hochberg method (False Discovery Rate) was applied to correct for any limitations related to performing multiple comparisons. Data are presented as means ± S.E.M. unless otherwise stated with statistical significance defined as a *P*-value ≤ 0.05.

RESULTS

Impact of acute exercise on monocyte subset distribution

Following flow cytometry analysis, the percentage of monocytes among PBMCs were unaltered in response to acute exercise relative to pre-exercise conditions (*pre-exercise:* 4.14 ± 1.99% vs. *post:* 4.09 ± 1.99%; *p* = 0.906). This finding suggests that adjusting for leukocytosis as evident by changes in the absolute number of monocytes within the PBMC (1.0×10^6 cells/mL)

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population is not necessary for subsequent statistical analyses. Prior to exercise, there was a significant difference in the percentage of monocyte subtypes ($F_{[2, 40.844]} = 2257.629$, $p \leq 0.001$; Figure 2A). More specifically, *classical* (CD14$^{+bright}$/CD16^{-}) monocytes accounted for 81.85 $\pm$ 4.72% of the total monocyte population, whereas the *intermediate* (CD14$^{+bright}$/CD16^{+dim}) and *non-classical* (CD14^{+dim}/CD16$^{+bright}$) monocyte subsets accounted for 4.68 $\pm$ 2.2% and 12.7 $\pm$ 4.90% of the total monocyte population, respectively.

In response to acute maximal exercise, the percentage of monocytes considered *classical* significantly decreased to 75.51 $\pm$ 6.88% of the total monocyte population ($p \leq 0.001$), whereas the *intermediate* and *non-classical* monocyte subsets significantly increased to 5.41 $\pm$ 2.57% ($p = 0.017$) and 18.18 $\pm$ 7.46% ($p \leq 0.001$) of the total monocyte population, respectively. Finally, the percent changes in monocyte subsets (pre to post exercise) were significantly different among all monocyte subsets ($F_{[2, 32.051]} = 24.968$; $p \leq 0.001$; Figure 2B), indicating that while the percentage of *classical* monocytes within the entire monocyte population decreased, the mobilization of *intermediate* monocytes during acute exercise was less pronounced compared to *non-classical* monocytes.

Impact of acute exercise on monocyte TLR4 expression

Prior to exercise, *classical* monocytes expressed a significantly lower TLR4 GMFI compared to both *intermediate* and *non-classical* monocyte subsets ($F_{[2, 45.221]} = 52.544$, $p \leq 0.001$; Figure 3A). In addition, *post hoc* analysis revealed no difference between the *intermediate* and *non-classical* monocyte subsets ($p = 0.363$).

In response to acute maximal exercise, the TLR4 GMFI significantly decreased on the total monocyte population compared to pre-exercise conditions ($p \leq 0.001$). In addition, TLR4

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expression significantly decreased on *classical* monocytes ($p = 0.001$) and the *intermediate* subset ($p \leq 0.001$), whereas TLR4 expression on the *non-classical* subset remained unaltered ($p = 0.079$). Similarly, while the percent change in TLR4 expression on c*lassical* monocytes was not different compared to *intermediate* monocytes, TLR4 expression decreased significantly more on *classical* and *intermediate* compared to the *non-classical* monocyte subset following exercise ($F_{[2, 34.200]} = 13.855$; $p \leq 0.001$; Figure 3B).

Impact of acute exercise on monocyte CD14 and CD16 surface expression

As expected, CD14 receptor expression was similar in *classical* and *intermediate* monocytes prior to exercise, and both expressed significantly greater levels of CD14 compared to *non-classical* monocytes ($F_{[2, 35.757]} = 1180.592$, $p \leq 0.001$; Figure 4A). In response to acute exercise, CD14 receptor expression was significantly lower on the total monocyte population ($p \leq 0.001$), and associated subsets compared to pre-exercise conditions; *classical* ($p \leq 0.001$), *intermediate* ($p = 0.014$), and *non-classical* ($p = 0.041$). Furthermore, the percent change in CD14 receptor expression was not different among *classical, intermediate*, and *non-classical* monocyte subsets ($F_{[2, 40.313]} = 0.916$, $p = 0.408$; Figure 4B).

Prior to exercise, CD16 receptor expression was significantly lower on *intermediate* compared to *non-classical* monocytes ($t_{[46]} = -8.452$, $p \leq 0.001$; Figure 4C). In response to exercise, CD16 significantly increased on the *intermediate* monocyte subsets ($p = 0.036$) and remained unaltered on *non-classical* monocytes ($p = 0.226$). However, the percent change in CD16 expression between *intermediate* and *non-classical* monocyte subsets was not different ($t_{[46]} = 1.397$, $p = 0.169$; Figure 4D).

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Impact of acute exercise on LPS-induced inflammatory cytokines

Compared to pre-exercise concentrations, *ex vivo* production of IL-6 and IL-10 from isolated PBMCs stimulated with LPS were significantly decreased in response to acute exercise ($t_{[24]} = 3.268$, $p = 0.002$; $t_{[24]} = 2.863$, $p = 0.005$, respectively; Figures 5A and B). Conversely, the ratio of IL-6 to IL-10 and the *ex vivo* production of the pro-inflammatory cytokine TNF-α were significantly increased in response to acute exercise compared to pre-exercise levels ($t_{[24]} = 4.612$, $p \leq 0.001$; $t_{[24]} = 2.128$, $p = 0.022$, respectively; Figure 5C and D).

Relationships among VO$_{2max}$, the percent changes in the phenotypic characteristics of monocytes, and their *ex vivo* production of inflammatory cytokines following acute exercise

VO$_{2max}$ was negatively associated with the percent change in the proportion of *intermediate* monocytes following acute exercise (r = -0.498, $p = 0.013$; Figure 6A). In addition, while VO$_{2max}$ was negatively associated with the percent change in CD14 receptor expression on *intermediate* monocytes following acute exercise (r = -0.484, $p = 0.022$; Figure 6B), VO$_{2max}$ tended to have a positive association with the percent change in CD16 receptor expression on *intermediate* monocytes (r = 0.322, $p = 0.058$). No other relationships among VO$_{2max}$ and the phenotypic changes of monocytes or their *ex vivo* production of inflammatory cytokines were observed.

No relationships were observed among the phenotypic changes on *classical* or *intermediate,* monocytes and changes in IL-6 and IL-10 production, the ratio of IL-6 to IL-10, or TNF-α production, indicating the production of these inflammatory cytokines may have occurred independent of the cardiorespiratory fitness and phenotypic changes observed on these monocyte subsets following acute exercise. To the contrary, the percent increase in TNF-α production from pre to immediately post exercise was positively associated with the percent change in the

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proportion of *non-classical* monocytes (r = 0.370, *p* = 0.038; Figure 6C) and negatively associated with the percent change in CD14 receptor expression on *non-classical* monocytes (r = -0.417, *p* = 0.048; Figure 6D). No other relationships were observed among the phenotypic changes on *non-classical* monocytes and changes in TNF-α production or other inflammatory cytokines.

DISCUSSION

This study examined the impact of the physical perturbation of acute maximal exercise on monocyte subset distribution and TLR4, CD14, and CD16 receptor expression patterns that determine the inflammatory phenotype of a standardized concentration of isolated monocytes, and their subsequent inflammatory response to *ex vivo* stimulation with LPS. Observations from this study support previous findings, demonstrating that acute exercise of various durations, intensities, and modes, including a short bout of maximal exercise to exhaustion, reduces the percentage of *classical* monocytes and increases the percentage of monocytes identified as *intermediate* and *non-classical* (Steppich et al., 2000; Hong and Mills, 2008; Simpson et al., 2009; Booth et al., 2010; Radom-Aizik et al., 2014). In addition, while other studies have shown that acute exercise reduces TLR4 expression on the monocyte population as a whole (Lancaster et al., 2005; Oliveira and Gleeson, 2010), data from the present study indicates that following acute maximal exercise, TLR4 expression is only lowered on *classical* and *intermediate* monocyte subsets and remains unaltered on the *non-classical* subset. Furthermore, the present study demonstrates that CD14 expression decreases on each monocyte subset, whereas CD16 expression increases on *intermediate*, but not the *non-classical* monocyte subset, suggesting that acute exercise preferentially mobilizes pro-inflammatory monocytes expressing lower levels of CD14 and higher levels of CD16 receptor expression. Likewise, given the associations with VO$_{2max}$, these responses may be augmented in

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intermediate monocytes in individuals with elevated cardiorespiratory fitness levels. To examine the potential impact of these phenotypic changes on innate immune function, isolated immune cells were stimulated *ex vivo* with LPS. Compared to pre-exercise concentrations, the LPS-stimulated production of the IL-6 (a pro- *and* anti-inflammatory cytokine) and the anti-inflammatory cytokine IL-10 decreased, whereas the ratio of IL-6 and IL-10 and production of the pro-inflammatory cytokine TNF-α increased. Thus, these findings indicate that acute maximal exercise alters the *cumulative* inflammatory phenotype of monocytes towards a pro-inflammatory milieu following *ex vivo* stimulation with LPS.

Chronic aerobic exercise training is considered anti-inflammatory and has been shown to reduce the pathology of pro-inflammatory diseases, including cardiovascular disease and metabolic dysfunction (Gleeson et al., 2011). In addition, the capacity of chronic aerobic exercise training to reduce TLR4 expression and shift the inflammatory phenotype of isolated immune cells towards an anti-inflammatory milieu has been supported (Timmerman et al., 2008; Gleeson et al., 2011). While acute exercise has also recently been suggested to be anti-inflammatory (Durrer et al., 2017), this notion has not been examined under a controlled culture environment using a standardized concentration of isolated PBMCs (1.0×10^6 cells/mL), in which the monocytes are the only cell type to respond to LPS stimulation (Wright et al., 1990). Early studies demonstrate that TLR4 surface expression decreases on the total circulating monocyte population immediately following prolonged submaximal exercise (1.5-2 hours in duration at 55% maximal work load or 75% VO$_{2peak}$, respectively) (Lancaster et al., 2005; Oliveira and Gleeson, 2010). More recently, studies have shown that TLR4 expression responds differently among monocyte subsets in response to exercise. For example, TLR4 expression decreases on *classical* monocytes and increases on the *intermediate* and *non-classical* monocyte subsets 1 hour into recovery from acute

submaximal (45 minutes of running at 75% VO$_{2max}$) and vigorous (60 km cycling time-trial) exercise (Simpson et al., 2009; Booth et al., 2010). Interestingly, the present study observed a decrease in TLR4 expression on *classical* and *intermediate* monocytes immediately following maximal exercise, but not on the *non-classical* monocyte subset, suggesting that combinations of exercise intensity and duration influence TLR4 expression differently among monocyte subsets.

The aforementioned findings suggest that the redistribution of monocyte subsets and the altered TLR4 surface expression patterns following acute exercise transiently alters the inflammatory phenotype of isolated monocytes in response to *ex vivo* stimulation with LPS. Lancaster et al. (2005) were the first to examine the impact of acute aerobic exercise on the inflammatory phenotype of monocytes. Researchers stimulated cultured whole blood samples with LPS prior to and immediately following prolonged submaximal aerobic exercise (1.5 hours at 55% peak work) in the heat (34° C), demonstrating that the LPS-induced production of intracellular IL-6 within the total monocyte population was attenuated post exercise (Lancaster et al., 2005). The inclination to interpret decreased IL-6 concentrations within monocytes as an anti-inflammatory response (i.e., suppression of the pro-inflammatory response) may potentially be misleading. For example, although elevated plasma IL-6 concentrations at rest are a biomarker of pro-inflammatory disease pathology (Van Gaal et al., 2006), Dr. B. K. Pedersen's laboratory (Copenhagen, Denmark) has demonstrated that systemic injection of IL-6 increases plasma IL-10 concentrations and inhibits the concomitant release of TNF-α into plasma following intravenous injection of LPS into healthy humans (Starkie et al., 2003; Steensberg et al., 2003). Similarly, the addition of IL-6 to cultured monocytes stimulated with LPS has been shown to downregulate TNF-α production (Aderka et al., 1989; Fattori et al., 1994) and enhance the polarization of macrophages towards and M2 (anti-inflammatory) phenotype by increasing anti-inflammatory cytokine

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production (including IL-10) (Fernando et al., 2014), lending to the consideration that IL-6 production from stimulated monocytes may potentially regulate the resultant inflammatory milieu during LPS stimulation. Thus, while is unclear whether or not the increased ratio of IL-6 to IL-10 in the present study reflects a *pro-inflammatory* response as previously suggested (Taniguchi et al., 1999), it is also possible that the attenuated net production of IL-6 that occurred independent of changes in *classical* monocyte distribution of phenotypic expression may have contributed to the observed suppression of IL-10. As a result, the decreased IL-6 and IL-10 may have enabled the production of TNF-α from *intermediate* and *non-classical* monocyte subsets to increase.

Bories et al. (2012) have demonstrated that the inflammatory phenotype of circulating monocytes determine their innate immune response upon infiltration and subsequent differentiation into resident macrophages inside tissue. Interestingly, repeated bouts of high intensity cycling exercise has been shown to enhance nuclear translocation of the nuclear factor (NF)-κB transcription factor, potentially upregulating numerous genes associated with an increased pro-inflammatory phenotype of isolated monocytes (Cuevas et al., 2005; Radom-Aizik et al., 2014). However, while acute exercise may decrease the quantity of cytokines produced per monocyte in response to LPS stimulation, as determined by intracellular staining of inflammatory cytokines within individual monocytes, the number of monocytes that are capable of producing cytokines in response to LPS stimulation has been shown to increase (Febbraio et al., 2002). Therefore, the positive relationship between the increased production of TNF-α and the proportion of *non-classical* monocytes supports the hypothesis that the mobilization of pro-inflammatory monocytes directly contributes to the net elevation of LPS-induced TNF-α production observed immediately following maximal exercise in the present study. Findings from this study further indicate that suppression of the anti-inflammatory response in monocytes (i.e., decreased IL-6 and

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IL-10 or increased ratio of IL-6 to IL-10) occurs in favor of a pro-inflammatory phenotype (i.e., increased TNF-α), and may reflect a positive response that is necessary to facilitate debris clearance from skeletal muscle damage (Chazaud et al., 2009), and to regulate vascular health and function following acute exercise (Radom-Aizik et al., 2014).

It is important to note that this plausible explanation does not fully account for changes in receptor expression patterns. For example, CD14 receptor expression decreased on both *intermediate* and *non-classical* monocytes. Given that CD14 is considered necessary to initiate an LPS-induced inflammatory response through TLR4 (Wright et al., 1990; Kim and Kim, 2014), it is unknown how this response on *non-classical* monocytes would potentially contribute to the elevated production of TNF-α. Nonetheless, *non-classical* monocytes are a more robust producer of TNF-α in response to LPS stimulation compared to *intermediate* monocytes (Wong et al., 2011). Thus, the enhanced mobilization of *non-classical* monocytes that maintained similar TLR4 expression levels, relative to the *intermediate* monocyte subset exhibiting lower TLR4 expression levels, may have contributed to the elevated *ex vivo* production of TNF-α observed immediately post exercise in this study. In addition, Pinheiro da Silva et al. (2007; 2008) demonstrate that CD16, independent of immunoglobulin G, interacts with LPS ligation to TLR4 to augment cellular TNF-α production. These findings indicate that the elevated expression of the CD16 receptor on *intermediate* monocytes may have also helped facilitate the enhanced production of TNF-α observed immediately following exercise. Finally, results from the present study suggest that elevated cardiorespiratory fitness levels may mediate the mobilization and phenotypic characteristics of *intermediate* monocyte subsets following acute exercise, potentially altering the pro-inflammatory immune response observed in the present study. Future studies should therefore consider targeting the function of specific receptors and include the utilization of polymyxin B, an

antibiotic that prevents LPS binding to TLR4 as a mechanistic control to verify that the inflammatory response is specific to LPS alone. Such studied would increase our understanding of the biological significance of these phenotypic changes and their potential role in regulating the innate immune response of monocytes following acute exercise in aerobically trained and untrained populations.

In summary, the results from this investigation support the hypothesis that acute maximal exercise alters the inflammatory phenotype of isolated monocytes exposed to *ex vivo* stimulation with LPS. While additional research is necessary to differentiate the impact of acute exercise on the inflammatory phenotype of each monocyte subset, the observed findings highlight potential mechanistic targets that may elucidate the role of acute and chronic exercise on the inflammatory phenotype of circulating monocytes. Results from such studies will further enhance our understanding of monocyte involvement in the regulation of the systemic and tissue specific innate immune response necessary for cardiovascular health.

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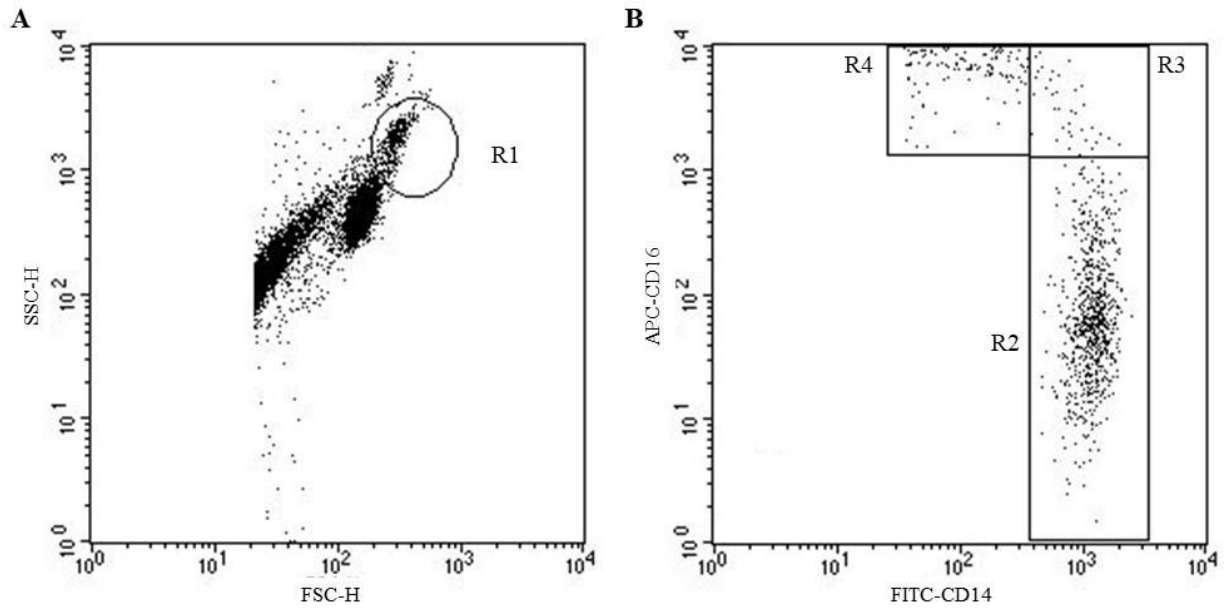
Figure 1. Representative flow cytometry dotplots demonstrating monocyte gating strategy. Panel A: Forward and 90° side scatter established primary gates for monocytes (R1) from isolated peripheral mononuclear cells. Panel B: Secondary gates were stablished against FITC-CD14 and APC-CD16 to identify monocytes according to Mukherjee et al. (2015). Monocyte subsets were identified according to CD14 and CD16 expression patterns. *Classical*: CD14$^{+bright}$/CD16^{-} (R2); *Intermediate*: CD14$^{+bright}$/CD16^{+dim} (R3); Non-classical: CD14^{+dim}/CD16$^{+bright}$ (R4).

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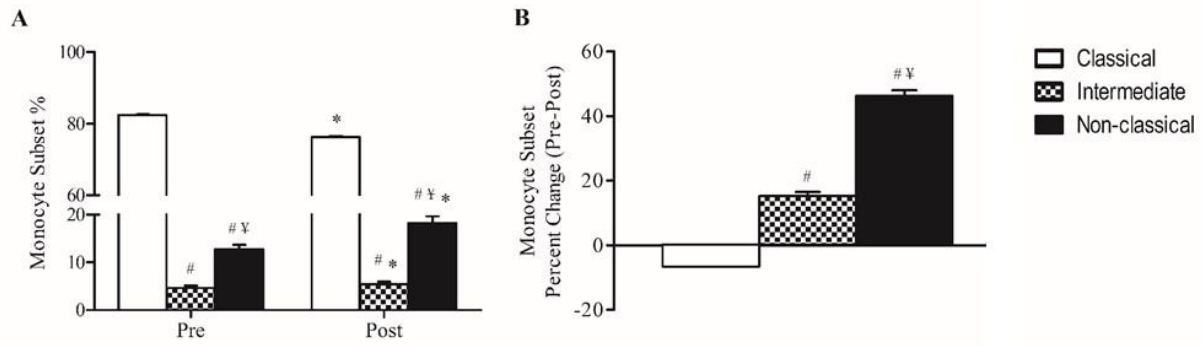
Figure 2. The percentage of monocytes identified as *classical, intermediate,* and *non-classical* prior to and immediately post exercise (Panel A), and the percent changes in each monocyte subsets from pre- to post-maximal exercise (Panel B). **Note:** # = difference in *classical* compared to *intermediate* and *non-classical* monocytes; ¥ = difference in *intermediate* compared to *non-classical* monocytes; * = difference compared to pre exercise (all $p \leq 0.05$). Data are presented as means ± SEM.
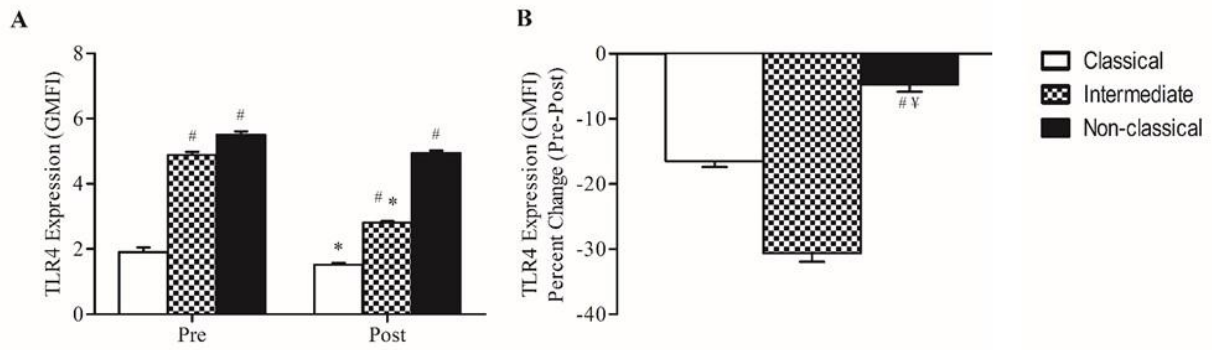
Figure 3. TLR4 expression on *classical, intermediate,* and *non-classical* prior to and immediately post exercise (Panel A), and the percent change in TLR4 expression from pre- to post-maximal exercise (Panel B). **Note:** # = difference in *classical* compared to *intermediate* and/or *non-classical* monocytes; ¥ = difference in *intermediate* compared to *non-classical* monocytes; * = difference compared to pre exercise ($p \leq 0.05$). Data are presented as means ± SEM.
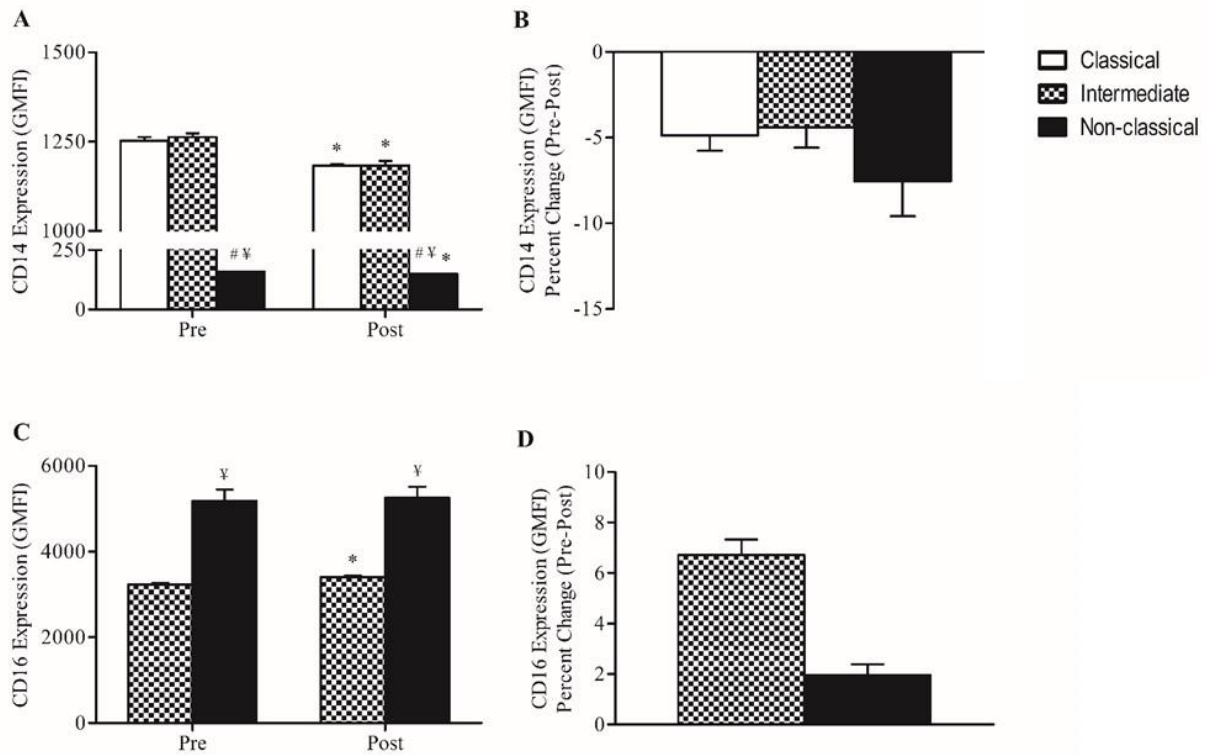
Figure 4. CD14 expression on *classical, intermediate,* and *non-classical* prior to and immediately post exercise (Panel A), and the percent change in in CD14 expression from pre- to post-maximal exercise (Panel B). CD16 expression on *classical, intermediate,* and *non-classical* prior to and immediately post exercise (Panel C), and the percent change in in CD16 expression from pre- to post-maximal exercise (Panel D). **Note:** # = difference in *classical* compared to *non-classical* monocytes; ¥ = difference in *intermediate* compared to *non-classical* monocytes; * = difference compared to pre exercise ($p \leq 0.05$). Data are presented as means ± SEM.
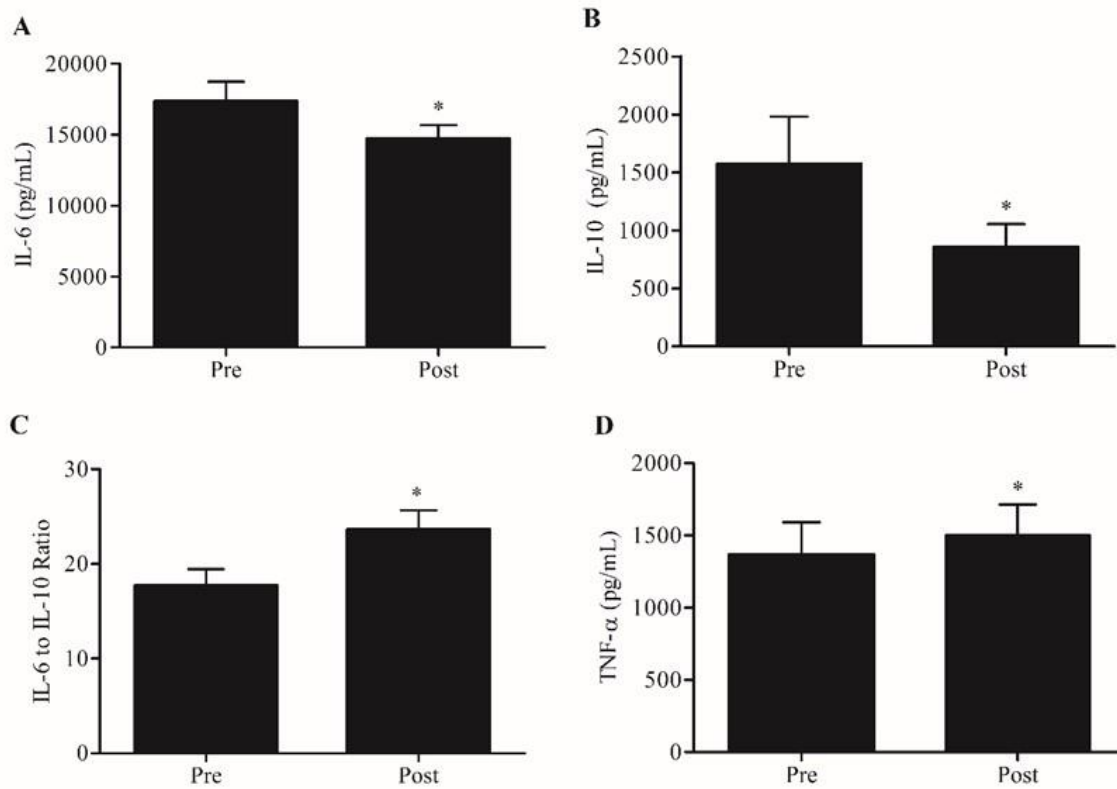
Figure 5. Changes in LPS-stimulated production of IL-6 (Panel A), IL-10 (Panel B), the ratio of IL-6 to IL-10 (Panel C), and TNF-α (Panel D) from isolated PBMCs prior to and immediately post exercise. **Note:** * = difference compared to pre exercise ($p \leq 0.05$). Data presented at mean ± SEM.

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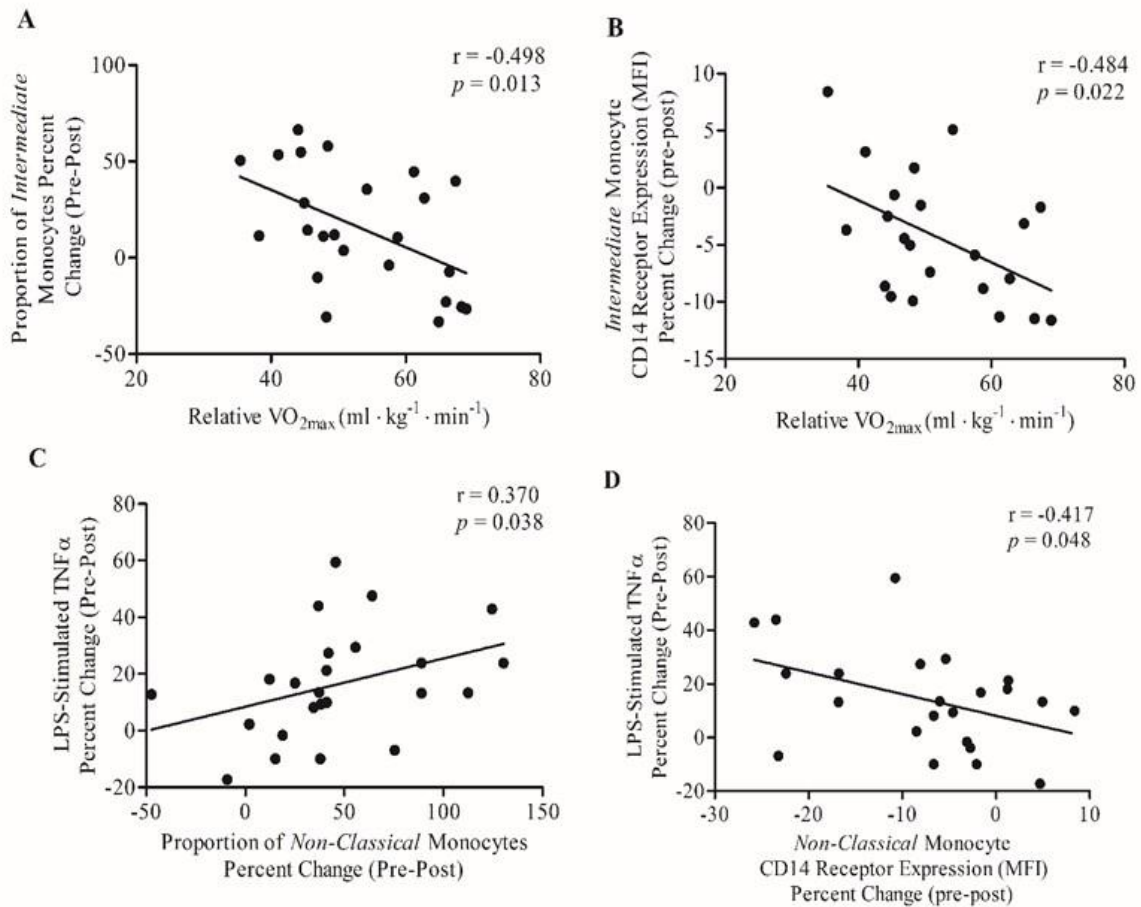
Figure 6. The association of relative VO_{2max} and the percent change in the proportion of *intermediate* monocytes (Panel A) and CD14 receptor expression (Panel B) and the association of the percent change in the proportion of *non-classical* monocytes (Panel C) and CD14 receptor expression (Panel D) with the percent change in LPS-stimulated TNF-α production from pre to post maximal exercise.

CHAPTER 4

AEROBIC FITNESS ALTERS THE CAPACITY OF MONONUCLEAR CELLS TO
PRODUCE PENTRAXIN 3 FOLLOWING MAXIMAL EXERCISE
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ABSTRACT

Purpose: Pentraxin 3 (PTX3) is a vital regulator of innate immune function. Although plasma PTX3 concentrations are elevated with aerobic fitness, differences in the cellular function of PTX3 remain unknown in aerobically trained and untrained subjects. **Methods:** Thirty individuals (trained = 15 and untrained = 15) participated in a bout of maximal exercise to examine *ex vivo* PTX3 production from isolated peripheral blood mononuclear cells (PBMCs) exposed to LPS or palmitate. The capacity of PTX3 to regulate the *ex vivo* production of inflammatory cytokines was also examined. **Results:** Elevated plasma PTX3 concentrations prior to exercise were positively associated with the percent change in plasma PTX3 concentrations from pre to immediately following acute exercise in all subjects, independent of cardiorespiratory fitness (VO_{2max}). In aerobically trained subjects only, the LPS- and palmitate-stimulated production of PTX3 from isolated PBMCs were reduced post exercise and this response was associated with lower plasma PTX3 concentrations at rest and in response to acute exercise. In addition, the PTX3-stimulated production of the anti-inflammatory cytokines IL-10 and TGF-β1 decreased following acute exercise, whereas IL-6 production remained unaltered in all subjects. However, the percent change in IL-6 production was positively associated with VO_{2max} in all subjects, and in trained subjects only, positively associated with elevated plasma PTX3 concentrations at rest and in response to acute exercise. **Conclusion:** These results suggest that aerobic fitness alters the capacity of mononuclear cells to produce PTX3 following maximal exercise, and may enhance its reciprocal role as a mediator of the innate immune response.

INTRODUCTION

Regular participation in aerobic exercise is well evidenced to reduce the pro-inflammatory profiles associated with cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) (Kahn

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et al., 2006; Van Gall et al., 2006; Gleeson et al. 2011). However, the identification of a novel circulating biomarker that may downregulate the inflammatory profiles associated with disease pathology following chronic aerobic exercise remains relatively unknown. Interestingly, plasma pentraxin 3 (PTX3) concentrations are elevated in aerobically trained compared to untrained individuals (Miyaki et al., 2011; 2012), and are rapidly increased (i.e., 2-4 weeks) upon the initiation of aerobic exercise training in previously sedentary individuals (Chu et al., 2012; Zempo-Miyaki et al., 2016). More importantly, these elevated plasma PTX3 concentrations are directly linked to the positive cardiovascular and metabolic adaptations that attenuate disease progression following aerobic exercise training (Miaki et al., 2011 2012; Chu et al., 2012; Zempo-Miyaki et al., 2016). In light of these findings, a closer examination of plasma PTX3 and its potential role in regulating inflammation in aerobically trained individuals may provide insight into the effectiveness of aerobic exercise as a behavioral strategy to reduce inflammatory disease, including CVD and T2DM.

PTX3 is characterized as an acute phase reactant that is vital to the regulation of neutrophil- and monocyte-mediated innate immune function (Norata et al., 2009; Deban et al., 2010; Bottazzi et al., 2016). Specifically, early neutrophil progenitors synthesize PTX3 and store readily available concentrations that are released into circulation following their activation as mature neutrophils with endotoxin (lipopolysaccharide [LPS]) (Imamura et al., 2007; Jaillon et al., 2007). Monocytes also produce and release PTX3 following exposure to LPS and the saturated fatty acid, palmitate (Imamura et al., 2007; Shiraki et al., 2016; Slusher et al., 2016b). Consequently, PTX3 concentrations are elevated in concert with other pro-inflammatory cytokines (i.e., interleukin [IL]-1β and tumor necrosis factor alpha [TNFα]), serving as a biomarker of CVD and T2DM (Rolph et al., 2002 Jenny et al., 2009; Yang et al., 2014). However, evidence has recently

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elucidated that PTX3 attenuates the development of heart disease, the formation of atherosclerotic plaques, and may help restore glucose homeostasis (Norata et al., 2009 Deban et al., 2010; Miyaki et al., 2014; Escobar-Morreale et al., 2017; Weiss et al., 2017). In addition, PTX3 prevents the excess transmigration of innate immune cells into the vascular endothelium during pro-inflammatory insult (Norata et al., 2009; Deban et al., 2010), inhibits the ligation of LPS to its transmembrane receptor, toll-like receptor 4 (TLR4), and downregulates the intracellular signaling pathways responsible for the transcription of pro-inflammatory cytokines by neutrophils and monocytes (Bozza et al., 2014; Shiraki et al., 2016). Our laboratory and others have further shown that PTX3 stimulates the production of interleukin-10 and other anti-inflammatory cytokines (i.e., transforming growth factor beta [TGF-β1]) from isolated monocytes (Shiraki et al., 2016; Slusher et al., 2016b) and facilitates the transition of monocyte-derived macrophages from an M1, pro-inflammatory, to an M2, anti-inflammatory, phenotype (Sun et al., 2015). These findings suggest that the anti-inflammatory capacity of PTX3 regulates the innate immune response and substantiates the role of PTX3 as a counterregulatory protein that protects against the pathology of pro-inflammatory disease, including CVD and T2DM.

Recently, our laboratory has also demonstrated that an acute bout of aerobic exercise (i.e., $\geq 70\%$ of an individual's cardiorespiratory fitness level [VO_{2max}]) increases plasma PTX3 concentrations in healthy, physically inactive individuals (Slusher et al., 2015; 2016a), yet the LPS-stimulated *ex vivo* production of PTX3 from isolated peripheral blood mononuclear cells (PBMCs) is significantly reduced immediately following acute exercise compared to pre-exercise stimulations (Slusher et al., 2017a). Although these findings signify that the acute exercise-induced plasma PTX3 response is independent of those produced by isolated PBMCs, this relationship has not been examined in aerobically trained individuals. Therefore, the purpose of this investigation

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was twofold. *Experiment 1:* We first examined how aerobic fitness influences the relationship of the exercise-induced plasma PTX3 response compared to the cellular production of PTX3 from LPS-stimulated whole blood and isolated PBMCs and palmitate-stimulated PBMCs in trained and untrained individuals. *Experiment 2:* We have also has recently shown that the reduced cellular production of PTX3 following acute aerobic exercise is associated with the decreased production of IL-6 and IL-10 in untrained individuals (Slusher et al., 2017a). As an extension of our past investigation, isolated PBMCs were stimulated with physiologically relevant concentrations of recombinant human PTX3 (rhPTX3; 500 pg/mL) to examine the likelihood that PTX3 mediates the *ex vivo* production of IL-6, IL-10, and activated (act)TGF-β1 from PBMCs isolated from aerobically trained and untrained individuals following acute exercise.

METHODS

Subjects

Thirty healthy aerobically trained (n = 15) and untrained (n = 15) subjects between 18 to 35 years old were recruited to participate in the study. Aerobically trained subjects were identified as those who regularly participated in 150 or more minutes of moderate-to-vigorous intensity aerobic exercise per week and untrained subjects were identified as those who participate in less than 150 minutes of moderate intensity physical activity per week, including aerobic, anaerobic, or resistance exercise, as determined by a 7-day physical activity recall (Craig et al., 2003). Prior to participation, subjects provided their informed consent and completed a medical history questionnaire. Subjects were excluded from participation if they had been previously diagnosed with inflammatory diseases/conditions, such as cardiovascular disease, chronic kidney or liver disease, diabetes, or were under the current administration of medication known to alter inflammatory and/or metabolic profiles. In addition, subjects who were users of tobacco products

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(cigarettes, cigars, chewing tobacco), and/or consumed an average of ten or more standard alcoholic beverages per week were excluded. The study was approved by the Institutional Review Board.

Exercise Testing Procedures

Subjects arrived at the laboratory at 6:00 a.m. following an overnight fast of at least eight hours. Subjects were also instructed to abstain from alcohol and caffeine intake for at least 24 hours and intense physical activity for at least 48 hours prior to the laboratory visit. Upon arrival, each subject confirmed their adherence to the previously mentioned instructions and were familiarized with all instruments and procedures. Immediately thereafter, height and weight were assessed using basic medical devices to quantify the subject's body mass index (BMI) by dividing the subjects weight in kilograms by the square of their height in meters. In addition, waist and hip circumferences were obtained by tape measure and body composition was assessed by bioelectrical impedance (BIA) methods (OMRON HBF-308, Bannockburn, IL, USA). Next, subjects were instructed to rest quietly for at least five minutes to obtain resting heart rate (HR) and blood pressure (BP) using HR monitor (Polar T31, Polar Electro, Kempele, Finland) and sphygmomanometer, respectively.

Subjects then participated in treadmill exercise test to assess maximal oxygen consumption (i.e., VO_{2max}) administered in gradation according to our laboratory's previously described protocol (Slusher et al., 2016a; 2017a). In brief, the test began with a 3-minute warm-up at 4.83 kilometers per hour and 0% grade. Following the warm-up, speed was increased to elicit 80% $\pm$ 5 bpm of the subject's age predicted maximal HR (APMHR; 220-age) within the first 2-minute stage (stage one). During the next two minutes (stage two), HR was allowed to reach steady-state. After

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the first four minutes, speed remained constant and the exercise intensity was elevated by increasing grade 2% every 2 minutes until the subject reached volitional exhaustion. In addition, HR and rating of perceived exertion (RPE; Borg's 15-point scale) were recorded during the final 15 seconds of every exercise stage. VO_{2max} was determined using the ParvoMedics Metabolic Measurement System (ParvoMedics, Sandy, UT, USA), with rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) assessed and averaged every 15 seconds to calculate respiratory exchange ratio (RER: VCO_2/VO_2). Criteria for attaining VO_{2max} included a plateau in O_2 consumption (defined as a failure to increase oxygen uptake by 150 mL/min with increased workload) or three of the following secondary criteria: RER $\geq$ 1.15, HR within 10 bpm of subject's APMHR, blood lactate $\geq$ 8.0 mmol/L (assessed be finger prick; LactateScout+; EKF Diagnostics; Cardiff, Wales), and an RPE $\geq$ 19.

Experiment 1

Assessment of Plasma PTX3 Concentrations

A 10 mL whole blood sample was drawn from each subject's antecubital vein prior to participation and immediately upon completion of the maximal exercise test using a 21G butterfly needle into a tube containing K_2 ethylenediaminetetraacetic acid (K_2EDTA) (BD Vacutainer, Franklin Lakes, NJ, USA). Whole blood samples were immediately centrifuged at 3000 rpm for 20 minutes at room temperature. The plasma supernatant was collected and centrifuged at 10,000g for 10 minutes at 4°C to obtain platelet-free plasma for the analysis of PTX3 in duplicate by enzyme-linked immunosorbent assay (ELISA) methods (R&D Systems, Minneapolis, MN, USA).

Cell Culture for ex vivo PTX3 Production from Whole Blood

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Two 6 mL whole blood samples were also collected prior to and immediately upon completion of the maximal exercise test as described above. One whole blood sample from each time point was stimulated with 10 ng/mL of LPS (Phenol extraction from *E. coli* 055:B5, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C with 5% CO_2 for 3 hours and manually inverted every 15 minutes. The unstimulated whole blood samples served as a time-course control for each time point. Following the incubation period, whole blood samples were centrifuged at 3000 rpm for 20 minutes at room temperature. The plasma supernatant was collected and centrifuged at 10,000g for 10 minutes at 4°C for the analysis of PTX3 by ELISA methods.

Cell Culture for ex vivo PTX3 Production from Isolated PBMCs

A 10 mL whole blood sample was collected prior to and immediately upon completion of the maximal exercise test and immediately centrifuged (3000 rpm for 20 minutes at room temperature). Following removal of the plasma supernatant, the leukocyte buffy coat was isolated and carefully layered over an equal volume of Ficoll-Paque (ρ = 1.077 g/mL; Sigma-Aldrich, St. Louis, MO, USA) in a conical tube and centrifuged at 400g for 30 minutes at room temperature. The isolated PBMCs layer was washed three times with saline, suspended in 1 mL RPMI 1640 media (Invitrogen, Carlsbad, CA, USA), and manually counted by hemocytometer. Counted PBMCs were then adjusted to a final volume of 1.0×10^6 cells/mL in RPMI 1640 media supplemented with 5% fetal bovine serum, 1% penicillin, and 1% streptomycin (Life Technologies, Carlsbad, CA, USA), aliquoted equally in a 96-well culture plate (Corning Incorporated, Corning, NY, USA), and stimulated with LPS (10 ng/mL) or palmitate (200 µmol/L; Sigma-Aldrich, St. Louis, MO, USA) as previously described (Slusher et al., 2016b; 2017a). Unstimulated cell samples served as a time-course control for each time point. Following a 24 hour

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incubation period at 37°C with 5% CO_2, culture supernatants were isolated for the analysis of PTX3.

Experiment 2

Cell Culture for ex vivo PTX3-Mediated Inflammatory Cytokine Production from Isolated PBMCs

Separate samples of isolated PBMCs (isolated as described above) were exposed to 500 pg/mL of rhPTX3 (R&D Systems, Minneapolis, MN, USA) containing 0.1% fatty acid free bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Slusher et al., 2016b). Unstimulated cell samples served as a time-course control for each time point. Following a 24-hour incubation period at 37°C with 5% CO_2, culture supernatants were isolated and analyzed for IL-6, IL-10, and actTGF-β1 by ELISA methods (R&D Systems, Minneapolis, MN, USA). Of note, actTGF-β1 concentrations were undetectable in three subjects and therefore not included in the data analysis.

Statistical Analyses

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS version 23.0). Independent *t*-tests were conducted to compare anthropometric and cardiorespiratory profiles between aerobically trained and untrained subjects. *Experiment 1:* A two group (trained and untrained) by two time point (pre- and immediately post-maximal exercise) repeated measures analysis of variance (rmANOVA) was used to examine the effects of acute aerobic exercise on plasma PTX3, LPS-stimulated PTX3 production from whole blood and isolated PBMCs, and palmitate-stimulated PTX3 production from isolated PBMCs. If the Mauchly's test indicated violation of the sphericity assumption, the degrees of freedom were

corrected by the Greenhouse-Geisser estimates. In addition, Pearson's correlations were utilized to examine the relationships among cardiorespiratory fitness (relative VO_{2max}) and plasma PTX3 and culture-stimulated PTX3 concentrations prior to (resting conditions) and in response to maximal exercise (percent change from pre- to immediately post-maximal exercise). *Experiment 2:* The impact of maximal exercise on the PTX3-stimulated production of inflammatory cytokines (IL-6, IL-10, and actTGF-β1) in aerobically trained and untrained subjects was assessed by rmANOVA, and the potential relationships of these variables with relative VO_{2max} and plasma PTX3 concentrations were examined with Pearson's correlations. All data are presented as means ± S.E.M. unless otherwise stated with statistical significance being defined as a *P*-value ≤ 0.05.

RESULTS

Subject Anthropometric Characteristics and Cardiovascular Measures

Baseline anthropometric characteristics and cardiovascular measures between trained and untrained subjects are reported in Table 1. Although no differences were observed in anthropometric characteristics, aerobically trained subjects presented with lower resting HR ($t_{[22.877]} = -3.816$, $p = 0.001$) and exhibited significantly greater absolute and relative VO_{2max} values ($t_{[28]} = 7.431$, $p < 0.001$; $t_{[28]} = 11.437$, $p < 0.001$, respectively) compared to untrained subjects.

Experiment 1

Plasma PTX3 Concentrations

Prior to participation in the maximal exercise protocol, no difference was observed in plasma PTX3 concentrations in aerobically trained compared to untrained subjects. In response to maximal exercise, repeated measures ANOVA revealed that plasma PTX3 concentrations

significantly increased to a similar extent in both aerobically trained and untrained subjects ($F_{[1, 28]} = 10.500$, $p = 0.003$; Figure 1A). In addition, elevated concentrations of plasma PTX3 prior to exercise were positively associated with the plasma PTX3 response to maximal exercise (r = 0.428, $p = 0.018$; Figure 1B). Finally, no associations were observed with plasma PTX3 concentrations and indices of cardiorespiratory fitness.

PTX3 Production Following Ex Vivo Stimulation of Whole Blood and Isolated PBMCs

Three separate culture experiments were performed to examine the relationship of plasma PTX3 concentrations with the cellular production of PTX3 from leukocytes stimulated with pro-inflammatory stimuli. Prior to exercise, no differences were observed in the production of PTX3 between aerobically trained and untrained subjects following LPS stimulation of whole blood and isolated PBMCs, or following palmitate stimulation of isolated PBMCs. In response to maximal exercise, the production of PTX3 from LPS stimulated whole blood was increased to a similar extent in both aerobically trained and untrained subjects ($F_{[1, 27]} = 4.524$, $p = 0.043$; Figure 2A), whereas the production of PTX3 tended to decrease more in aerobically trained compared to untrained subjects following LPS stimulation of isolated PBMCs ($F_{[1, 28]} = 2.935$, $p = 0.098$; Figure 2B). Likewise, there was a significant decrease in palmitate stimulated PTX3 production in aerobically trained compared to untrained subjects following maximal exercise ($F_{[1, 28]} = 6.404$, $p = 0.017$; Figure 2C). Notably, no differences were observed in the constitutive release of PTX3 from any culture sample at rest or in response to maximal exercise, as assessed in the unstimulated time course control samples (data not shown).

To further assess how cardiorespiratory fitness may have influenced these responses, the relationship of relative VO_{2max} and culture-stimulated PTX3 production under all three conditions

were assessed prior to and in response to maximal exercise (percent change from pre- to immediately post-maximal exercise). As a result, no associations were observed between relative VO_{2max} and culture-stimulated PTX3 production prior to participation in maximal exercise. To the contrary, relative VO_{2max} was negatively associated with the percent change in LPS-stimulated PTX3 production from whole blood (r = -0.641, $p < 0.001$; Figure 2D), but not isolated PBMCs (r = -0.272, $p = 0.146$; Figure 2E). In addition, relative VO_{2max} was negatively associated with the percent change in palmitate-stimulated PTX3 production from isolated PBMCs (r = -0.406, $p = 0.026$; Figure 2F).

Next, the associations of plasma PTX3 with the cellular production of PTX3 exposed to pro-inflammatory stimuli was assessed prior to and immediately following maximal exercise. Consistent with our previous reports, plasma PTX3 concentrations appear independent to those produced by whole blood and isolated PBMCs following exposure to pro-inflammatory stimuli (Slusher et al., 2016b; 2017a). However, given the apparent role of cardiorespiratory fitness (relative VO_{2max}) as a mediator of cellular PTX3 production, the relationship of plasma PTX3 and culture-stimulated PTX3 concentrations were examined independently in aerobically trained and untrained subjects. No relationships were observed in plasma PTX3 concentrations with LPS-stimulated PTX3 production from whole blood samples in aerobically trained or untrained subjects. To the contrary, plasma PTX3 concentrations were positively associated with the percent change in LPS- and palmitate-stimulated PTX3 production from PBMCs at rest (r = 0.610, $p = 0.016$; r = 0.621, $p = 0.014$, respectively; Figures 3A and B) and in response to maximal exercise (r = 0.539, $p = 0.038$; r = 0.578, $p = 0.024$, respectively; Figure 3C and D) in aerobically trained, but not untrained, subjects, even after controlling for relative VO_{2max}.

Experiment 2

PTX3-Stimulated Inflammatory Cytokine Production from Isolated PBMCs

To examine the inflammatory response of isolated PBMCs following exposure to elevated concentrations of plasma PTX3 after participation in maximal exercise, isolated PBMCs were cultured in the presence of 500 pg/mL of PTX3. Prior to exercise, no differences were observed in the production of IL-6 and IL-10 between aerobically trained and untrained subjects following PTX3 stimulation, whereas the PTX3-stimulated production of actTGF-β1 was significantly lower in aerobically trained compared to untrained subjects ($t_{[18.056]}$ = -2.102, p = 0.050). Following maximal exercise, the PTX3-stimulated production of IL-6 from isolated PBMCs was unaltered in aerobically trained subjects and tended to decrease in untrained subjects ($F_{[1, 28]}$ = 2.596, p = 0.118; Figure 4A), whereas the production of IL-10 and activated TGF-β1 significantly decreased to a similar extent in aerobically trained and untrained subjects ($F_{[1, 28]}$ = 9.564, p = 0.004; $F_{[1, 25]}$ = 40.015, p < 0.001, respectively; Figures 4B and C).

To further assess how cardiorespiratory fitness may have influenced the inflammatory response of isolated PBMCs following PTX3 stimulation, the relationship of relative VO$_{2max}$ and PTX3-stimulated IL-6, IL-10, and actTGF-β1 production were assessed prior to and in response to maximal exercise. While no associations were observed between relative VO$_{2max}$ and the PTX3-stimulated production of inflammatory cytokines prior to participation in maximal exercise, a positive association was observed between relative VO$_{2max}$ with the percent change in PTX3-stimulated IL-6 production from isolated PBMCs (r = 0.382, p = 0.041; Figure 4D), but not IL-10 (r = -0.172, p = 0.363; Figure 4E). In addition, the association observed between relative VO$_{2max}$ with the percent change in PTX3-stimulated actTGF-β1 production approached significance (r = -0.325, p = 0.098; Figure 4F).

Finally, the relationships of plasma PTX3 and the production of PTX3-stimulated IL-6, IL-10, and actTFG-β1 were examined independently in aerobically trained and untrained subjects. Plasma PTX3 concentrations were positively associated with the production of PTX3-stimulated IL-6 prior to participation in maximal exercise in aerobically trained subjects only (r = 0.555, p = 0.032; Figure 5A). Similarly, plasma PTX3 concentrations prior to and in response to maximal exercise (percent change from pre- to immediately post-maximal exercise) were positively associated with the percent change in PTX3 stimulated IL-6 production in aerobically trained subjects (r = 0.662, p = 0.007; r = 0.625, p = 0.013, respectively; Figures 5B and C), even after controlling for relative VO_{2max}. No associations were observed with PTX3-stimulated IL-10 and actTGF-β1 prior to or in response to maximal exercise.

DISCUSSION

The primary purpose of this investigation was to examine how aerobic fitness influences the systemic and cellular PTX3 response, and in turn, the capacity of PTX3 to mediate the innate immune response following maximal exercise. Previous research in South Korean and Japanese subjects of all ages demonstrates that regular participation in aerobic exercise improves cardiovascular and metabolic health by increasing resting concentrations of plasma PTX3 (Miyaki et al., 2011; 2012; Chu et al., 2012; Zempo-Miyaki et al., 2016). To the contrary, results from the present investigation are consistent with those presented by Huang et al. (2014), demonstrating that aerobic fitness may not influence plasma PTX3 concentrations prior to or in response to maximal exercise in young, healthy subjects from the United States. These findings further suggest that factors related to geographical location and/or genetic influences may alter the capacity of aerobic exercise training to influence plasma PTX3 concentrations. Nevertheless, our results

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demonstrate that elevated concentrations of plasma PTX3 prior to participation in acute exercise predict a greater systemic increase in plasma PTX3 concentrations immediately upon completion from maximal exercise, independent of aerobic training status or cardiorespiratory fitness level.

We then exposed whole blood and isolated PBMC samples to LPS, and separately to palmitate, to examine whether or not regular participation in aerobic exercise alters the PTX3 production capacity of immune cells as a potential mechanism that may contribute to elevated PTX3 concentrations in plasma following physiological stress (i.e., maximal exercise). As a result, the LPS-stimulated production of PTX3 from whole blood increased equally in both aerobically trained and untrained subjects, and despite the apparent influence of cardiorespiratory fitness, these responses remained independent of the plasma PTX3 response in aerobically trained and untrained subjects. Flow cytometry analysis has demonstrated that intracellular PTX3 concentrations within neutrophils are lower following acute bouts of strenuous exercise, supporting their role as a key contributor to the plasma PTX3 response (Nakajima et al., 2010). These results further suggest that the increased production of PTX3 from LPS-stimulated whole blood, compared to no change in the control conditions, may have resulted from the mobilization of circulating monocytes capable of releasing PTX3.

To remove the potential contribution of neutrophils and the influence of leukocytosis on the cellular PTX3 response, we examined the PTX3 production capacity of a standardized concentration of isolated PBMCs (1.0×10^6 cells/mL), in which monocytes are the only cell source to contribute to increased concentrations of PTX3 following inflammatory stimulation (Maina et al., 2009). Our laboratory has previously demonstrated that participation in an acute bout of submaximal aerobic exercise (70% of VO_{2max}) reduced the net production of PTX3 from isolated PBMCs exposed to LPS in healthy, sedentary individuals (Slusher et al., 2017a). To the contrary,

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the present study observed no differences across time or between subject groups in response to acute maximal exercise, and despite the tendency for the LPS-stimulated production of PTX3 to be lower in aerobically trained compared to untrained subjects ($p = 0.098$), there was no relationship between this response and cardiorespiratory fitness level. Similar to the influence of exercise intensity on the plasma PTX3 response (Nakajima et al., 2010), these results indicate that the greater intensity of exercise (maximal vs. submaximal) may help maintain the capacity of monocytes to synthesize and release PTX3 following exposure to elevated levels of endotoxin observed during recovery from acute exercise. Therefore, additional analysis regarding the subset of monocyte (i.e., *classical, intermediate,* and *non-classical*) responsible for PTX3 production and the impact of exercise intensity on their PTX3 producing capacity would provide a necessary explanation for gaps in the literature.

Furthermore, our laboratory recently demonstrated that exposure of PBMCs to palmitate also increases the *ex vivo* production of PTX3 in healthy individuals (Slusher et al., 2016b). Thus, we examined the influence of aerobic fitness on this cellular response prior to and immediately following completion of maximal exercise. Notably, maximal exercise suppressed the palmitate-stimulated PTX3 production capacity of PBMCs in aerobically trained, but not untrained, subjects, a response that was negatively associated with elevated levels of cardiorespiratory fitness in all subjects. While endotoxin and saturated fatty acids elicit an inflammatory response through similar mechanisms (i.e., TLR4) (Pålsson-McDermott and O'Neill, 2004; Shi et al., 2006; Sindhu et al., 2016), results from the present investigation indicate that aerobic exercise training decreases the cellular responsiveness of monocytes exposed to palmitate following acute physiological stress. These results suggest that additional signaling mechanisms may be involved in palmitate-stimulated PTX3 production, and are differentially altered in aerobically trained and untrained

subjects following acute exercise. Likewise, the positive associations of plasma PTX3 concentrations prior to and in response to acute maximal exercise with the decreased production of PTX3 from isolated PBMCs exposed to LPS and palmitate observed in the trained subject group suggests that aerobic exercise may influence the capacity of PTX3 to mediate the innate immune response in monocytes.

To corroborate the aforementioned posit, experiment two stimulated isolated PBMCs with physiologically relevant concentrations of PTX3 (500 pg/mL) as determined in previously reported studies (Miyaki et al., 2011; Slusher et al., 2015; 2016b). Compared to pre exercise conditions, maximal exercise significantly reduced the PTX3-stimulated capacity of isolated PBMCs to produce the anti-inflammatory cytokines IL-10 and actTGF-β1. While no differences were observed across time or between subject groups in the PTX3-stimualted production of IL-6, there was a positive relationship observed between elevated cardiorespiratory fitness levels and the percent change in IL-6 production from pre to immediately post maximal exercise. Upon further investigation, plasma PTX3 concentrations were positively associated with PTX3-stimulated IL-6 production prior to and in response to maximal exercise in aerobically trained subjects only. IL-6 is typically considered a pro-inflammatory cytokine associated with increased pathology of cardiovascular and metabolic disease (Kahn et al., 2006; Van Gaal et al., 2006). However, other studies have shown that IL-6 exerts a variety of anti-inflammatory functions that parallel those of PTX3, including the inhibition of LPS-stimulated pro-inflammatory cytokines and the augmentation of anti-inflammatory cytokine production (Fattori et al., 1994; Steensberg et al., 2003; Shiraki et al., 2016). In addition, both IL-6 and PTX3 have been shown to facilitate the polarization of monocyte-derived macrophages from an M1 to an M2 phenotype (Fernando et al., 2014; Pucci et al., 2014; Sun et al., 2015), supporting their anti-inflammatory function.

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Furthermore, our laboratory has recently demonstrated that despite a positive relationship between the cellular production of PTX3 and IL-6 following LPS stimulation in healthy, aerobically untrained subjects, these responses are independent of plasma PTX3 concentrations (Slusher et al., 2017a). Given that IL-6 does not stimulate the cellular production of PTX3 (Breviario et al., 1992), it may be that the PTX3 response to pro-inflammatory stimulation (e.g., IL-1β, TNF-α, LPS, and palmitate) helps facilitate a reciprocal anti-inflammatory response (i.e., IL-6, IL-10, and actTGF-β1) from monocytes residing within the local microenvironment.

Our results support the collective body of literature to suggest that aerobic exercise and elevated levels of aerobic fitness mediate the function of PTX3 at rest and in response to acute exercise. As an extension upon these findings, elevated levels of aerobic fitness also appear to alter the cellular production of PTX3 following exposure to endotoxin and saturated fatty acids prior to and in response to acute maximal exercise, potentially enhancing the reciprocal capacity of PTX3 to mediate an anti-inflammatory response from monocytes. Future investigations should include a more thorough examination of the intrinsic cellular function of PTX3 to provide additional insight into the role of PTX3 as a mechanistic target to prevent and treat CVD and T2DM following habitual participation of aerobic exercise.

Table 1. Subject anthropometric characteristics, cardiovascular measures, and indices of cardiorespiratory fitness

Variable	Trained (n = 15)	Untrained (n = 15)	P value
Age (y)	25.27 ± 4.574	23.13 ± 3.502	0.163
Weight (kg)	69.420 ± 6.347	70.273 ± 8.709	0.761
Height (m)	1.790 ± 0.052	1.778 ± 0.082	0.621
BMI	21.681 ± 1975	22.185 ± 1.744	0.465
Waist (cm)	75.493 ± 3.098	77.333 ± 5.093	0.676
Hip (cm)	95.407 ± 3.461	96.740 ± 5.795	0.451
W:H	0.791 ± 0.027	0.780 ± 0.035	0.454
Body Fat Percentage	11.080 ± 4.096	13.440 ± 0.035	0.163
Resting HR (bpm)	60.200 ± 5.348	70.467 ± 8.943	*0.001
Resting SBP (mmHg)	117.333 ± 8.269	113.333 ± 8.997	0.215
Resting DBP (mmHg)	78.400 ± 9.891	77.067 ± 8.581	0.696
Absolute VO_{2max} (L·min^{-1})	4.367 ± 0.406	3.145 ± 0.491	*< 0.001
Relative VO_{2max} (mL·kg^{-1}·min^{-1})	63.047 ± 4.290	44.793 ± 4.450	*< 0.001

Note: The * indicates a significant difference between trained and untrained subjects at baseline ($p < 0.05$). Data are presented as means ± S.D. BMI, body mass index; W:H, waist-to-hip ratio; HR, Heart Rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; VO_{2max}, maximal oxygen uptake
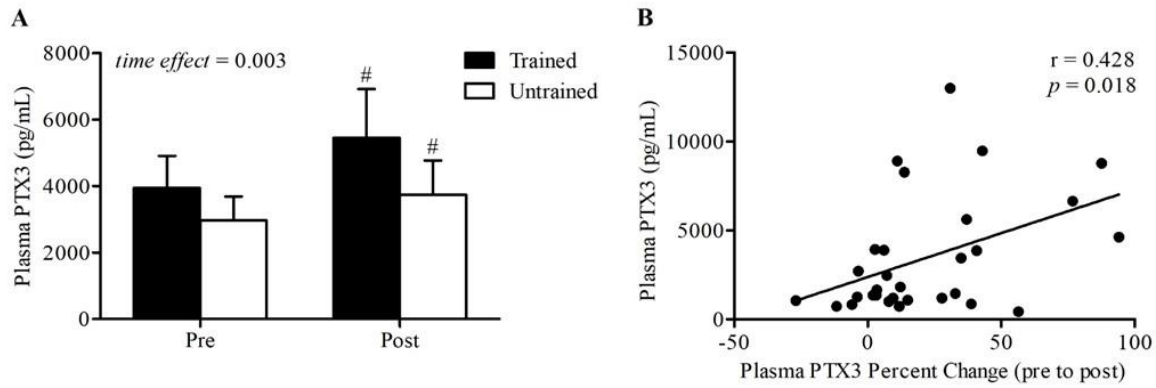
Figure 1. Plasma PTX3 concentrations at prior to and in response to acute maximal exercise in aerobically trained and untrained subjects (panel A). The relationship of plasma PTX3 concentrations prior to maximal exercise and the percent change in plasma PTX3 concentrations from pre to post acute maximal exercise (panel B). # indicates a significant difference in plasma PTX3 concentrations relative to pre exercise ($p \leq 0.05$).
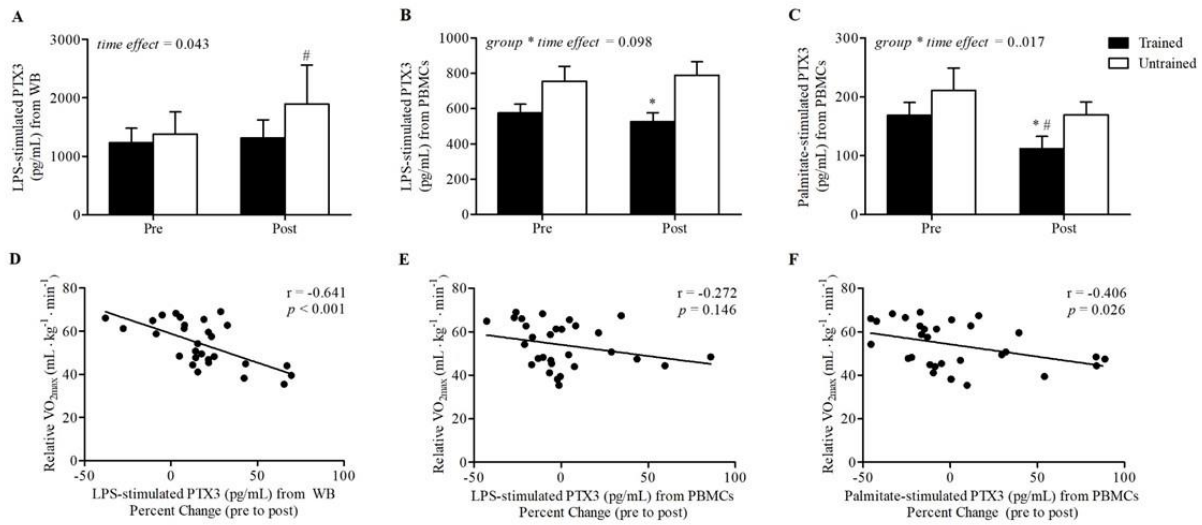
Figure 2. PTX3 productions levels following LPS stimulation of whole blood (panel A) and isolated PBMCs (panel B), or following palmitate stimulation of isolated PBMCs (panel C) in aerobically trained and untrained subjects. The relationship of relative VO_{2max} and culture-stimulated PTX3 production under all three conditions in response to maximal exercise (percent change from pre to immediately post maximal exercise) (panels D-F). * indicates a significant difference in PTX3 production levels between aerobically trained and untrained subjects ($p \leq$ 0.05). # indicates a significant difference in plasma PTX3 concentrations relative to pre exercise ($p \leq 0.05$).

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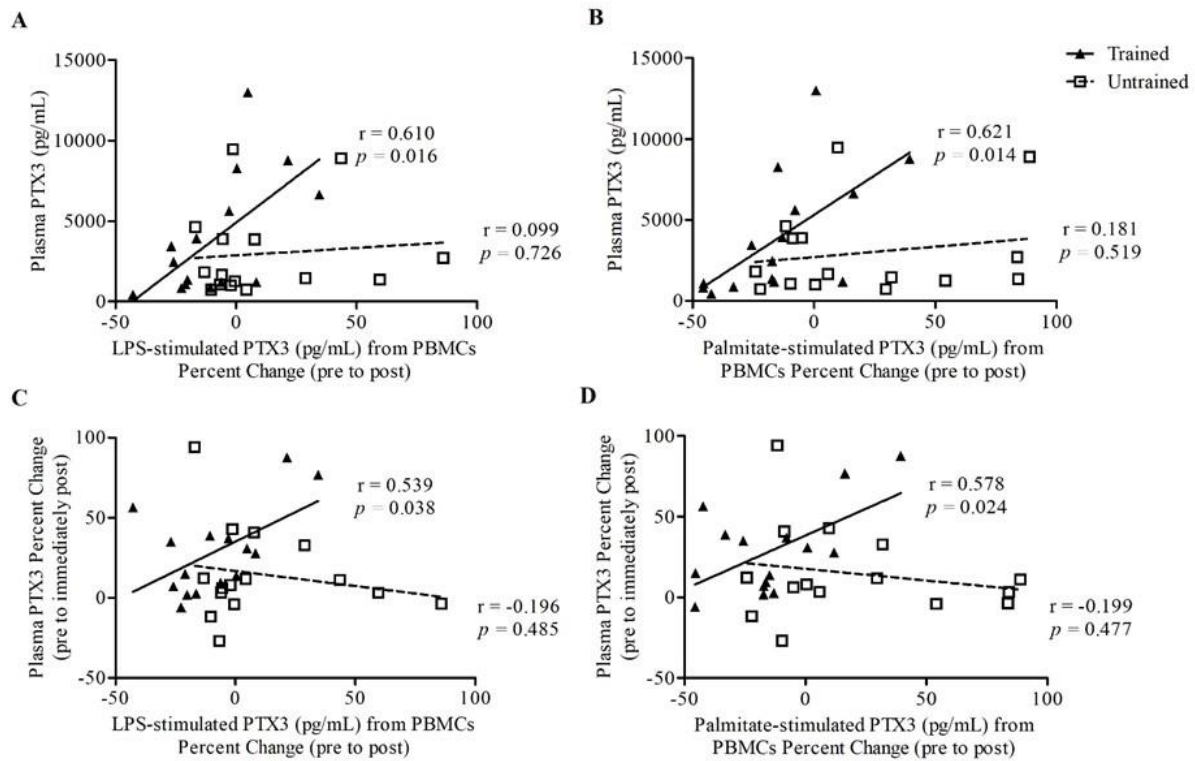
Figure 3. The relationship of plasma PTX3 concentrations prior to and in response (percent changed) to acute maximal exercise with PTX3 productions levels following LPS and palmitate stimulation of isolated PBMCs assessed separately in aerobically trained (closed triangle) and untrained (open square) subjects (panels A-D).
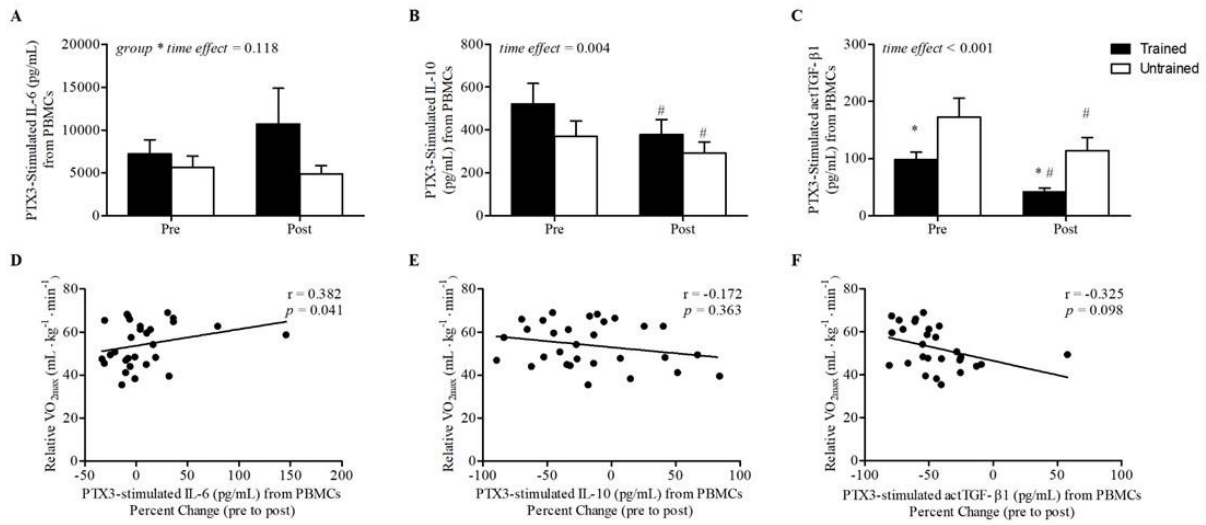
Figure 4. PTX3-stimulated productions of the inflammatory cytokines IL-6 (panel A), IL-10 (panel B), and TGF-β (panel C) from PBMCs isolated from aerobically trained and untrained subjects. The relationship of relative VO_{2max} and PTX3-stimulated inflammatory cytokine production in response to maximal exercise (percent change from pre to immediately post maximal exercise) (panel D-F). * indicates a significant difference in PTX3 production levels between aerobically trained and untrained subjects ($p \le 0.05$). # indicates a significant difference in plasma PTX3 concentrations relative to pre exercise ($p \le 0.05$).

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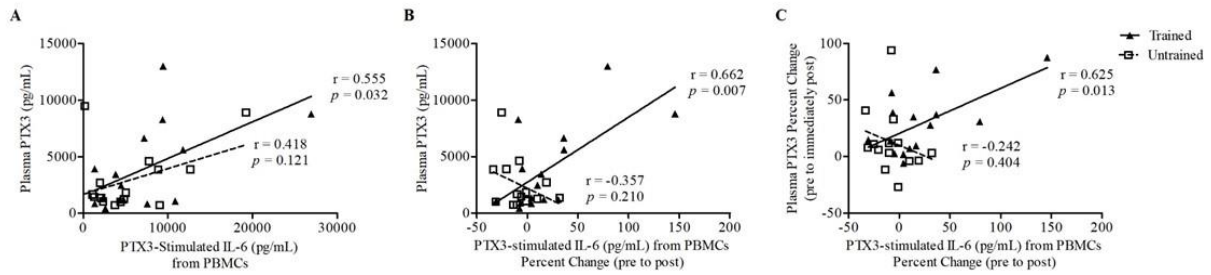
Figure 5. The relationship of plasma PTX3 concentrations prior to and in response (percent changed) to acute maximal exercise with PTX3-stimulated IL-6 productions levels from of isolated PBMCs assessed separately in aerobically trained (closed triangle) and untrained (open square) subjects (panels A-C).

CHAPTER 5

INFLAMM-AGING IS ASSOCIATED WITH LOWER PLASMA PTX3 CONCENTRATIONS AND AN IMPAIRED CAPACITY OF PBMCS TO EXPRESS hTERT mRNA FOLLOWING LPS STIMULATION

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ABSTRACT

Age-related elevations in pro-inflammatory cytokines, known as *inflamm-aging*, are associated with shorter immune cellular telomere lengths. **Purpose:** This study examined the relationship of plasma PTX3 concentrations, a biomarker of appropriate immune function, with telomere length in middle-aged and young adults. In addition, the capacity of isolated PBMCs to express a key mechanistic component involved in maintaining longer telomere lengths, human telomerase reverse transcriptase (hTERT), was examined following cellular stimulation with LPS, PTX3, and PTX3+LPS. **Methods:** In 15 middle-aged (40-64 years of age) and 15 young adults (20-31 years of age), PTX3 and inflammatory cytokines (i.e., IL-6, IL-10, TGF-β, and TNF-α) were measured in plasma, telomere lengths were measured from isolated PBMCs, and hTERT mRNA expression and inflammatory protein production was examined following exposure of PBMCs with LPS, PTX3, and PTX3+LPS. **Results:** Aging was accompanied by the accumulation of centrally located visceral adipose tissue, independent of changes in body weight and BMI, and chronic alterations in the systemic inflammatory milieu (decreased plasma PTX3 and TGF-β [$p = 0.009$; $p = 0.012$, respectively]; increased plasma TNF-α [$p = 0.044$]). In addition, telomere lengths were shorter in middle-aged compared to young adults ($p = 0.011$), and associated with increased age and body fat percentages (r = -0.404 $p = 0.027$; r = -0.427, $p = 0.019$, respectively). Finally, the capacity of PBMCs to express hTERT mRNA following cellular stimulation was impaired in middle-aged compared to young adults ($p = 0.033$), and negatively associated with telomere lengths (r = 0.353, $p = 0.028$). **Conclusions:** These findings suggest that aging is linked to lower plasma PTX3 concentrations and demonstrate that the impaired capacity of PBMCs to express hTERT mRNA may contribute to age-related shortening of telomere lengths.

INTRODUCTION

Aging is accompanied by the chronic, low-grade elevation of circulating pro-inflammatory cytokines (i.e., interleukin 6 [IL-6] and tumor necrosis factor alpha [TNF-α]) that typically manifests during middle age (40-64 years of age) (Álvarez-Rodríguez et al., 2012; Morrisette-Thomas et al., 2014). This phenomenon has recently been termed *inflamm-aging* (Franceschi et al., 2000). While the causes of *inflamm-aging* are numerous (Franceschi and Campisi, 2014), the pathology has been hypothesized to derive from the natural loss of subcutaneous adipose tissue (SAT) and the accumulation of centrally located visceral adipose tissue (VAT) that occurs in the absence of weight gain or changes in body mass index (BMI) (Kuk et al., 2009; Zamboni et al., 2014). Within the stromal vascular fraction of VAT, the number of T cell lymphocytes increase, and resident monocyte-derived macrophages are polarized towards an M1, pro-inflammatory, phenotype (Wu et al., 2007; Cartier et al., 2009; Starr et al., 2009; Lumeng et al., 2011). As a result, the increased production and secretion of circulating pro-inflammatory cytokines contribute to the increases risk of premature morbidity and mortality from age-related diseases, including cardiovascular disease (CVD) and metabolic dysregulation (Kahn et al., 2006; Van Gaal et al., 2006).

Telomeres maintain the "youthful" function of immune cells, and shortened telomere lengths are a biological marker of cellular aging (Benetos et al., 2001). More specifically, telomeres are gene-poor regions located at the ends of chromosomes and are formed by thousands of hexameric $5'(TTAGGG)_n3'$ repeats that protect chromosomes from degradation and end-to-end-fusion (Blackburn and Gall, 1978; Moyzis et al., 1988). Although telomere lengths shorten as a natural consequence of aging (Frenck et al., 1998; Iwama et al., 1998), the persistent exposure of circulating immune cells (i.e., T cell lymphocytes and monocytes) to age-related pro-inflammatory

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profiles is thought to enhance the rate of telomere attrition (Merino et al., 2011; O'Donovan et al., 2011; Hearps et al., 2012; Jurk et al., 2014). Once telomeres reach a critically shortened length, immune cells enter an irreversible state of replication-induced cellular senescence (Hermann et al., 2001; d'Adda di Fagana et al., 2003; Zou et al., 2004). These aged immune cells exhibit a senescent-associated secretory phenotype (SASP) that further elevate levels of pro-inflammatory cytokines at rest and following exposure to inflammatory stimulants, such as the gram-negative endotoxin lipopolysaccharide (LPS) (Coppé et al., 2008; Minamino et al., 2009; Merino et al., 2011). More worrisome may be that cellular senescence also spreads from cell to cell and other organ systems, including adipose tissue (Tchkonia et al., 2010; Nelson et al., 2012), exacerbating the progression of the *inflamm-aging* phenotype associated with age-related diseases (Minamino et al., 2009; Merino et al., 2011; Baker et al., 2012; Jurk et al., 2014; Spyridopoulos et al., 2016).

Prior to senescence, leukocytes express human telomerase reverse transcriptase (hTERT), the rate-limiting component and surrogate marker of the telomerase enzyme that is recruited to facilitate the generation of new telomeric DNA necessary to maintain the length and structural integrity of telomeres (Grieder et al., 1989; Yu et al., 1990; Kyo et al., 1998; Akiyama et al., 2002). In response to acute inflammatory challenge, hTERT mRNA expression is significantly increased in the THP-1 macrophage cell line following stimulation with LPS and other pro-inflammatory stimulants (Gizard et al., 2011). Interestingly, basic cell line research demonstrates that the capacity of leukocytes to express hTERT mRNA decreases with each cell replication (Röth et al., 2003), suggesting that the progressive and persistent inflammatory assault observed with *inflamm-aging* may also impair the capacity of leukocytes to express hTERT mRNA in middle-aged compared to young adults. Consequently, the decreased capacity of leukocytes to express hTERT has been shown to be a central factor associated with telomere length shortening and the induction

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of cellular senescence (Gizard et al., 2011; Ramunas et al., 2015). However, the hypothesis that age-related changes in adiposity, independent of changes in body weight and BMI, are associated with chronic, low-grade elevations of pro-inflammatory cytokines which alter the length of telomeres and associated mechanisms (i.e., hTERT) has yet to be thoroughly investigated in healthy human adults. Such gaps within the literature highlight the need to examine hTERT as a potential cellular target which links the mechanistic consequences of *inflamm-aging* to telomere length-dependent replication-induced cellular senescence.

Pentraxin 3 (PTX3) is a counterregulatory protein that is expressed and secreted from isolated leukocytes in concert with various inflammatory proteins (e.g., IL-6, IL-10, TGF-β, and TNF-α) following LPS-stimulation (Lee et al., 1990; Imamura et al., 2007; Maina et al., 2009; Slusher et al., 2017a). Although PTX3 is commonly associated with acute inflammatory insult, PTX3 is required to prevent over activation of the inflammatory signaling pathway and elevated concentrations of plasma PTX3 are considered an indicator of appropriate immune function in young, healthy adults (Yamaskai et al., 2009; Shiraki et al., 2016; Slusher et al., 2016a; 2017a; 2017b). In addition, Pavanello et al. (2017) have recently demonstrated that elevated concentrations of plasma PTX3 are associated with longer telomere lengths in healthy middle-aged adults. Less is known about whether or not plasma PTX3 concentrations are altered as a consequence of *inflamm-aging*, and how PTX3 may potentially be associated with the pro-inflammatory milieu that impairs telomeric-associated mechanisms. Therefore, age-related changes in plasma PTX3 concentrations and its relationship with telomere length will be examined in middle-aged (40-64 years of age) and young adults (20-31 years of age). In addition, the capacity of PTX3 to mediate the LPS-induced inflammatory response and hTERT mRNA expression in PBMCs isolated from middle-aged and young adults will also be examined.

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METHODS

Research Participants

A total of thirty healthy young (n = 15; between 20 and 31 years of age) and middle-aged (n = 15; between 40 and 64 years of age) adults were recruited to participate in this study. All subjects presented with a BMI associated with a reduced risk of CVD according to Stevens et al. (1998). Prior to their enrollment, each subject provided their informed consent and completed a medical history questionnaire to verify that they had not been previously diagnosed with any cardiovascular, metabolic, renal, liver, pulmonary, asthmatic, rheumatic, or other inflammatory disease/condition, were not currently under the administration of medication known to alter their inflammatory or metabolic profiles, or within the past 10 years, had not been diagnosed with any cancer requiring radiation or chemotherapy treatment. Furthermore, subjects who were currently using or have used tobacco products within the past six months or who consumed > 10 alcoholic beverages per week on average were excluded from participation in the study. Finally, all subjects completed a 7-day International Physical Activity Questionnaire to verify that they participated in ≤ 150 minutes of moderate to vigorous physical activity per week (Craig et al., 2003), and were therefore classified as physically inactive according to the American College of Sports Medicine (ACSM, 2013). The University's Institutional Review Board approved the study.

Laboratory Procedure

Subjects arrived at the laboratory between 6:30 and 8:30 o'clock in the morning following an overnight fast of at least eight hours. In addition, each subject abstained from alcohol, caffeine intake, and moderate-to-vigorous physical activity for at least 24 hours prior to their participation. Immediately upon arrival, anthropometric measures were obtained, including an assessment of

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height and weight to determine BMI in kilograms per meters squared (kg/m^2), waist and hip circumferences to determine waist-to-hip ratio (W:H ratio), body fat percentage (BF%) evaluated by air displacement plethysmography from measured lung volume using the BOD POD (Cosmed; Chicago, IL, USA), and sagittal diameter of the abdominal region at the level of the L4/L5 vertebrae to determine an indirect measurement of VAT (Kvist et al., 1988). Each subject was then provided a quite resting place for at least 10 minutes to assess resting heart rate and blood pressure.

Plasma Protein Analysis

Whole blood samples were drawn into K$_2$EDTA tubes (BD Vacutainer, Franklin Lakes, NJ) from each subject's antecubital vein under quiet resting conditions. Blood samples were immediately centrifuged at 3000 RPM for 20 minutes at room temperature. Plasma supernatants were collected and stored at -80°C in cryopreservation tubes for the future analysis of plasma PTX3 and TGF-β by enzyme-linked immunosorbent assay (ELISA) kits and plasma IL-6, IL-10, and TNF-α using high-sensitivity ELISA kits according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

DNA Isolation and Measurement of Relative Telomere Length

The remaining buffy coat was collected, brought to a 5 mL volume with saline, and diluted PBMCs were layered over equal volumes of Ficoll-Paque (ρ = 1.077 g/mL; Sigma-Aldrich, St. Louis, MO) for a 30-minute centrifugation at 400·g at room temperature. Isolated PBMCs were washed with saline three times, and pelleted cells were lysed in TRIzol (Thermo Fisher, Waltham, MA, USA) for the isolation DNA according to manufacturer's instructions. Isolated DNA was quantified by spectrophotometry using the NanoDrop 2000 (Thermo Scientific, Wilmington, DE,

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USA). A 15 µL reaction of 2x SYBR green master mix (QuantaBio, Beverly, MA, USA), nuclease free water, target primers (Integrative DNA Technologies, Skokie IL, USA), and a 15 µg sample of total DNA were utilized to quantify relative telomere length by assessing the ratio of telomere repeats (Tel 1b: 270 nM 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′; Tel 2b: 900 nM 5′-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′) to the 36B4 reference gene (36B4u: 300 nM 5′-CAGCAAGTGGGAAGGTGTAATCC-3′; 36B4d: 500 nM 5′-CCCATTCTATCATCAACGGGTACAA-3′) (T/S ratio) using PCR methodologies in triplicate (Cawthon et al., 2002). Amplification conditions for RNA detection were set to heat activation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 54°C for 2 minutes. For 36B4 PCR, primers were incubated as above, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 58°C for 1 minute. Standard curves and dissociation curves for each primer set were performed to ensure equal efficiencies and single product formation, respectively. Relative T/S ratios were calculated according to Cawthon et al. (2002). Finally, the intra-assay coefficient of variation between triplicate samples was 2.24 and 1.31% for the telomere and 36B4 gene, respectively.

RNA Isolation and Measurement of hTERT mRNA Expression

A separate sample of isolated PBMCs were manually counted by hemocytometer, and cultured in complete RPMI 1640 media supplemented with 5% fetal bovine serum and 1% penicillin and streptomycin in the presence of LPS (10 ng/mL from *E. coli* O55:B5; Sigma Aldrich, St. Louis, MO, USA), recombinant human (rh) PTX3 (100 ng/mL; R&D Systems, Minneapolis, MN, USA), or a combination of rhPTX3 and LPS (initiated by a 30-minute pre-incubation period with rhPTX3). Unstimulated samples served as a time-course control. Stimulated cell samples

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were incubated at 37°C with 5% CO_2 for a 4-hour period at a final concentration of $2 \cdot 10^6$ in a 6-well culture plate. Following the completion of the 4-hour culture period, PBMCs were homogenized with TRIzol (Thermo Fisher, Waltham, MA, USA) and centrifuged for 2 minutes at $16,000 \cdot g$ in QIAshredder mini spin columns (Qiagen, Hilden, Germany) to reduce DNA contamination from cell extracts. RNA was then fully isolated using TRIzol methods according to manufacturer's instruction and quantified by spectrophotometry using the NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). A total of 1 µg of RNA was synthesized into cDNA according to manufacturer's instructions (QuantaBio, Beverly, MA, USA), and a 15 µL reaction of 2x SYBR green master mix (QuantaBio, Beverly, MA, USA), nuclease free water, target primers (Integrative DNA Technologies, Skokie IL, USA), and a 10 µg sample of cDNA were aliquoted into a 96-well plate. Each sample was analyzed for changes in hTERT mRNA (500 nM F: 5′-TACGGCGACATGGAGAACAAG-3′; 500 nM R: 5′-GGGCATAGCTGAGGAAGGTTT-3′) expression against the reference gene GAPDH (200 nM F: 5′-GAAGGTGAAGGTCGGAGTC-3′; 200 nM R: 5′-GAAGATGGTGATGGGATTTC-3′) by qPCR methodologies in triplicate using a CFX96 TOUCH thermal cycler and analyzed using CFX software (Bio-Rad, Hercules, CA, USA). Amplification conditions for RNA detection were set to heat activation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds. All melting curve analysis were performed between 65°C and 95°C and relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001).

Ex Vivo Stimulation of Isolated PBMCs

Protein concentrations of PTX3, IL-6, IL-10, TGF-β, and TNF-α were determined from cell culture supernatants from the 4-hour culture period, and separately, from a 24-hour culture period ($1·10^6$ cells/mL) in duplicate by ELISA methods according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical Analyses

Data analyses were performed using the Statistical Package for the Social Sciences (SPSS version 24.0). Independent *t*-tests were conducted to determine potential differences in anthropometric profiles (height, weight, BMI, waist and hip circumferences and W:H ratio, BF%, and sagittal diameter), cardiovascular health (resting heart rate, blood pressure, and mean arterial pressure), plasma PTX3, IL-6, IL-10, TGF-β, and TNF-α concentrations, and relative telomere lengths (T/S ratio) at rest. In addition, a two group (young adult and middle-aged adult) by four condition (time-course control, LPS, PTX3, and PTX3+LPS) repeated measures analysis of variance (rmANOVA) was utilized to examine differences in hTERT mRNA expression levels following *ex vivo* stimulation of PBMCs following the 4-hour stimulation period in young adult and middle-aged subjects. Likewise, differences in the capacity of PBMCs to produce PTX3 and the inflammatory cytokines IL-6, IL-10, TGF-β, and TNF-α between young adult and middle-aged subjects in response to 4- and 24-hour stimulation periods were examined by a two group by two condition (PTX3) or four condition (i.e., IL-6, IL-10, TGF-β, and TNF-α) rmANOVA. If the Mauchly's test indicated a violation of sphericity assumptions, the degrees of freedom were corrected by using Greenhouse-Geisser estimates. Finally, Pearson's correlations were utilized to examine the relationship among each variable, with application of the Benjamini-Hochberg

method (False Discovery Rate) to correct for any limitations related to performing multiple comparisons. Statistical significant being defined as a p-value ≤ 0.05.

RESULTS

Subject Descriptive Characteristics

Subject characteristics are presented in Table 1. Although these data demonstrate that no differences in body weight ($t_{[28]} = 0.087$, $p = 0.932$) or BMI ($t_{[28]} = 1.811$, $p = 0.081$) were observed between middle-aged and young adults, waist circumferences ($t_{[28]} = 2.166$, $p = 0.019$), W:H ratio ($t_{[28]} = 3.088$, $p = 0.003$), BF% ($t_{[28]} = 4.054$, $p \leq 0.0010$), and sagittal diameter ($t_{[28]} = 4.081$, $p \leq 0.001$) were significantly greater middle-aged adults. Likewise, while no associations were observed between age and body weight (r = 0.027, $p = 0.886$) or BMI (r = 0.292, $p = 0.117$), age was positively associated with waist circumference (r = 0.398, $p = 0.029$), W:H ratio (r = 0.531, $p = 0.003$), BF% (r = 0.549, $p = 0.002$), and sagittal diameter (r = 0.674, $p \leq 0.001$; Figure 1A-F). These data support the hypothesis that the natural, healthy aging process is accompanied by the increased accumulation of centrally located VAT that occurs in the absence of weight gain or changes in BMI.

Furthermore, women were shorter, weighed less, had lower BMI, smaller waist and hip circumferences and W:H ratios, and greater BF% compared to men. However, no other gender differences were observed among the remaining variables examined.

Plasma PTX3 and Senescent-Associated Inflammatory Cytokines

Baseline concentrations of plasma PTX3 were significantly lower in middle-aged compared to young adults ($t_{[23.897]} = 2.851$, $p = 0.009$; Figure 2A). Although no differences in

plasma IL-6 or IL-10 were observed ($t_{[28]} = 1.384$, $p = 0.089$; $t_{[28]} = 1.303$, $p = 0.102$, respectively), plasma concentrations of the pro-inflammatory cytokine TNF-α were significantly greater ($t_{[28]} = 1.767$, $p = 0.044$) and concentrations of the anti-inflammatory cytokine TGF-β were significantly lower in middle-aged compared to young-adults ($t_{[28]} = 2.381$, $p = 0.012$; Figures 2B-E). No associations between plasma PTX3 concentrations and the senescent-associated inflammatory cytokines were observed.

PBMC Relative Telomere Length

Relative telomere lengths (T/S ratio) analyzed from isolated PBMCs were significantly shorter in middle-aged compared to young adults ($t_{[28]} = 2.421$, $p = 0.011$; Figure 3A) and negatively associated with increased age and BF% (r = -0.404 $p = 0.027$; r = -0.427, $p = 0.019$, respectively; Figures 3B and C). While relative telomere lengths also tended to be negatively associated with circulating concentrations of plasma TNF-α (r = -0.323, $p = 0.082$), no associations were observed with plasma PTX3 or other senescent-associated inflammatory cytokines.

hTERT mRNA Expression

In response to *ex vivo* stimulation of isolated PBMCs with LPS, PTX3, and PTX3+LPS, the capacity of middle-aged adults to express hTERT mRNA relative to the time-course control condition was significantly impaired under all culture conditions compared to young adults ($F_{[3, 84]} = 3.053$, $p = 0.033$; Figure 4A). Although PTX3 did not alter the capacity of LPS to express hTERT mRNA, pre-incubation of PBMCs with PTX3 for 30 minutes prior to LPS stimulation was sufficient to significantly reduce hTERT mRNA expression relative to the time-course control conditions in middle-aged adults only. Finally, changes in the LPS-stimulated hTERT mRNA

expression (relative to the time-course control condition) were negatively associated age (r = -0.446, p = 0.007; Figure 4B) and positively associated relative telomere lengths (r = 0.353, p = 0.028; Figure 4C).

Ex Vivo Production of PTX3 and Senescent-Associated Inflammatory Cytokines from Isolated PBMCs

The capacity of isolated PBMCs to produce PTX3 following 4- and 24-hour *ex vivo* stimulation with LPS were similar middle-aged and young adults ($F_{[1, 28]}$ = 116.521, $p \leq 0.001$; $F_{[1, 28]}$ = 158.38, $p \leq 0.001$; Figures 5A and B). Following the 4-hour stimulation period, the overall production of IL-6 was significantly greater in middle-aged compared to young adults ($F_{[2.002, 56.043]}$ = 5.094, p = 0.009; Figure 5C). However, these differences were present only in PTX3 and PTX3+LPS culture conditions. Furthermore, only a condition effect was observed for IL-6 production following the 24-hour stimulation period ($F_{[1.299, 36.358]}$ = 25.91, $p \leq 0.001$; Figure 5D). In addition, only a condition effect was observed for the production of TNF-α ($F_{[1.602, 44.849]}$ = 41.682, $p \leq 0.001$; $F_{[2.201, 61.630]}$ = 57.628, $p \leq 0.001$, respectively; Figures 5E and F) and IL-10 (C $F_{[1.261, 35.31]}$ = 5.771, $p \leq 0.016$; $F_{[2.293, 64.211]}$ = 75.961, $p \leq 0.001$, respectively: Figures 5G and H) following the 4- and 24-hour stimulation periods. Finally, there was a significant increase in TGF-β following the 4-hour stimulation period in both groups ($F_{[2.064, 57.786]}$ = 8.605, $p \leq 0.001$; Figure 5I), whereas PTX3 significantly increased TGF-β following the 24-stimulation period in young adults only ($F_{[1.722, 48.206]}$ = 3.912, p = 0.032; Figure 5J).

DISCUSSION

This study is the first to demonstrate that the natural, healthy aging process is associated with the accumulation of centrally located VAT, independent of changes in body weight and BMI, and basal alterations in the systemic inflammatory milieu (decreased plasma PTX3 and TGF-β; increased plasma TNF-α). In addition, results from the present study suggest that the persistent exposure of immune cells to a pro-inflammatory milieu may alter the length of telomeres by impairing the capacity of isolated PBMCs to express hTERT mRNA following cellular stimulation with LPS, PTX3, and PTX3+LPS in middle-aged compared to young adults. Changes in body composition with age naturally favor the reduction of skeletal muscle mass, the reduced expansion of SAT, and the increased accumulation of centrally located VAT (Kuk et al., 2009; Kohara, 2014). In addition, alterations of adipocyte paracrine signals induce a pro-inflammatory phenotype of resident monocyte-derived macrophages that contribute to elevated concentrations of circulating pro-inflammatory cytokines (Lumeng et al, 2011). This phenomenon, *inflamm-aging*, was first proposed in 2000 by Claudio Franceschi, stating that the human immune system evolved as a protective mechanism for those who had historically survived up to 40-50 years of age (Franceschi et al., 2000; De Martinis et al., 2005). However, life expectancy has significantly increased in recent history (Riley, 2001; Oeppen and Vaupel, 2002), and it is suggested that the persistent inflammatory stimuli from an aged immune system functioning beyond its evolutionary limits progresses age-related inflammatory disease through the shortening of telomere lengths (Franceschi et al., 2000; Albright et al., 2016). In support of this hypothesis, elevated plasma concentrations of the pro-inflammatory cytokine TNF-α and decreased plasma concentrations of the anti-inflammatory cytokine TGF-β were observed in middle-aged compared to young adults. These findings are consistent with Álvarez-Rodríguez et al. (2012), and the lack of difference in plasma IL-6 concentrations support the notion that plasma IL-6 is the "cytokine for gerontologists"

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with age-related differences becoming more apparent in individuals ≥ 60 years of age (Ershler, 1993; Franceschi et al., 2000; Álvarez-Rodríguez et al., 2012).

The present study also examined the impact of age on resting plasma PTX3 concentrations. Elevated plasma PTX3 concentrations are considered an immunological biomarker associated with the decreased risk of age-related CVD and metabolic dysfunction in otherwise healthy adults (Yamaskai et al., 2009; Osorio-Conles et al., 2011; Slusher et al., 2016a). Similarly, although Osorio-Conles et al. (2011) demonstrated that PTX3 mRNA expression is increased and positively associated with the presence of pro-inflammatory protein expression in mature adipocytes isolated from VAT, plasma PTX3 concentrations are decreased in obese compared to normal-weight individuals. Given the similarities among obesity- and age-related changes in adipose tissue (Tzanetakou et al., 2012), and the positive role that PTX3 has been shown to play in the regulation of appropriate immune function and prevention of CVD and metabolic disease (Salio et al., 2008; Norata et al., 2009; Deban et al., 2010; Slusher et al., 2016a; 2017a; 2017b), plasma PTX3 concentrations were expectedly lower in middle-aged compared to young adults. Therefore, additional research is warranted to determine whether or not lower plasma PTX3 concentrations during middle-age are related to morphological changes of VAT or an impairment of neutrophils, the primary cellular source of PTX3, to synthesize and store PTX3 throughout their maturation (Jallion et al., 2007; Alonso-Fernández et al., 2008).

Telomere lengths have previously been shown to be inversely associated with systemic concentrations of plasma IL-6 and TNF-α in elderly adults (70-79 years old) and positively associated with plasma PTX3 concentrations in adult nurses between 18 and 65 years of age (Pavanello et al., 2017). The lack of a relationship between plasma IL-6 and TNF-α concentrations and telomere lengths in the present study suggest that the link between age-related changes in

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systemic inflammatory cytokine concentrations and telomere length may become more pronounced during the later stages of life when cellular senescence and age-related diseases are more prevalent. In addition, the lack of a relationship of plasma PTX3 with telomere length may be due to the physically inactive nature of the subject groups in the present study. More specifically, Pavanello et al. (2017) compared PTX3 and telomere lengths in day-shift and night-shift nurses. While no differences in plasma PTX3 were observed between these two groups, telomere lengths were significantly longer in night-shift nurses, who were younger and more physically active, suggesting that the impact of physical activity on telomeres may alter the relationship of PTX3 with telomere lengths (Miyaki et al., 2011; Loprinzi, 2015; Loprinz et al., 2015). Larger scale population studies may provide more insight into variables that influence the utilization of PTX3 as an indicator of cellular health.

Data from the present study also demonstrate that the capacity of isolated PBMCs to express hTERT is impaired in healthy, middle-aged adults. Gizard et al. (2011) have previously shown that LPS stimulation of human macrophages significantly increases hTERT mRNA expression. However, the capacity of cells to express hTERT decreases as cells approach their maximum number of replications known as the Hayflick limit (Hayflick and Moorhead, 1961; Röth et al., 2003), Ramunas et al. (2015) has recently revealed that the delivery of modified mRNA encoding hTERT rapidly extends telomere lengths, increases cellular replication capacity, and thus, prevents to onset of replication-induced cellular senescence in various human cell lines. Therefore, the inability of middle-aged adults to express hTERT following inflammatory challenge may reveal an early consequence of aging that contributes to the cascade of telomere-dependent cellular senescence, and consequently, the development of age-related disease.

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To examine whether or not circulating immune cells exhibit characteristics of cell SASP, isolated PBMCs were stimulation with LPS for 4- and 24-hour periods. The lack of significant differences in the PBMC-mediated inflammatory response following *ex vivo* LPS stimulation suggests that increased central adiposity, but not circulating monocytes, the primary cell type within PBMCs to respond to LPS stimulation (Wright et al., 1990; Alles et al., 1994; Maina et al., 2009), are the major contributor to the pro-inflammatory milieu observed in middle-aged compared to young adults. Similarly, the lack of an elevated inflammatory response characteristic of the SASP in middle-aged compared to young adults indicates that replication induced-cellular senescence has yet to manifest.

In light of recent findings by Shiraki et al. (2016), demonstrating that pre-incubation of macrophages and endothelial cells with 100 ng/mL of PTX3 can reduce the production of pro-inflammatory proteins (e.g., TNF-α) and increase TGF-β, the ability of PTX3 to alter the LPS-stimulated hTERT mRNA expression and the production of inflammatory cytokines in PBMCs isolated from middle-aged and young adults was also examined. Interestingly, while hTERT mRNA expression following the pre-incubation of PBMCs with PTX3 was not different compared to LPS alone in middle-aged or young adults, the addition of PTX3 was sufficient to suppress hTERT mRNA expression relative to the time-course control condition in middle-aged adults only. In addition, the pre-incubation of PBMCs with PTX3 did not differentially alter the LPS-stimulated production of IL-6 and TGF-β between subject groups, whereas TNF-α production was enhanced in both groups following the 4-hour stimulation period, and in response to the 24-hour stimulation period, PTX3 enhanced TNF-α production in middle-aged adults and suppressed IL-10 production in young adults. Although these findings suggest that although PTX3 may enhance the "early" phase pro-inflammatory innate immune response (i.e., 4-hour), the capacity of PTX3

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to alter the pro- and anti-inflammatory arm of the "late" phase (i.e., 24-hour) innate immune response may differ with increased age. Clearly, additional research on the influence of PTX3 to regulate hTERT and other telomeric-related mechanisms responsible for maintaining telomere length across the lifespan is warranted.

In conclusion, hTERT undoubtedly assumes a central role as a vital regulator of telomere maintenance, and is potentially a novel cellular target that links the mechanistic consequences of *inflamm-aging* to telomere length prior to the development of telomere-dependent cellular senescence. However, hTERT only explained about 12.5% of the variance in telomere length in the present study. These findings highlight the complexity of telomeric biology that is tightly controlled by a variety of highly orchestrated and interconnected mechanisms, including the six-protein shelterin complex and telomeric repeat-containing RNA, which regulate telomere length through the recruitment of the telomerase enzyme (Da-Silva et al., 2010; Redon et al., 2010; Schmidt et al., 2014; Wang et al. 2015). Therefore, future studies should consider utilizing age-related transcriptomic changes within telomere stabilizing genes and explore their non-telomeric functions within immune cells to help elucidate their impact on the sequela of age-related inflammatory disease pathology at the cellular level.

Table 1. Subject anthropometric and cardiovascular measures

Variable	Young Adult (n = 15)	Middle Aged (n = 15)	*P* value
Sex (M/F)	7/8	3/12	0.130
Age (y)	25.27 ± 3.26	57.27 ± 6.75	≤ 0.001*
Weight (kg)	66.54 ± 13.39	66.91 ± 9.94	0.932
Height (m)	1.73 ± 0.11	1.68 ± 0.07	0.141
BMI (kg·m^{-2})	22.00 ± 2.49	23.50 ± 2.03	0.081
Waist (cm)	73.65 ± 8.87	80.15 ± 7.51	0.039*
Hip (cm)	99.84 ± 7.04	99.26 ± 5.95	0.809
W:H ratio	0.74 ± 0.06	0.81 ± 0.07	0.005*
Body Fat Percentage (%)	19.41 ± 6.21	28.55 ± 8.43	0.005*
Sagittal Diameter (cm)	20.08 ± 2.22	23.01 ± 2.04	0.002*
Resting HR (bpm)	67.07 ± 9.54	66.07 ± 12.66	0.809
Resting SBP (mmHg)	113.20 ± 9.375	120.13 ± 18.59	0.211
Resting DBP (mmHg)	74.40 ± 11.54	75.73 ± 10.14	0.739
MAP	87.33 ± 10.00	88.53 ± 13.25	0.782

Note: The * indicates a significant difference between middle-aged and young adults ($p <$ 0.05). Data are presented as means ± S.D. BMI, body mass index; W:H, waist-to-hip ratio; HR, Heart Rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure
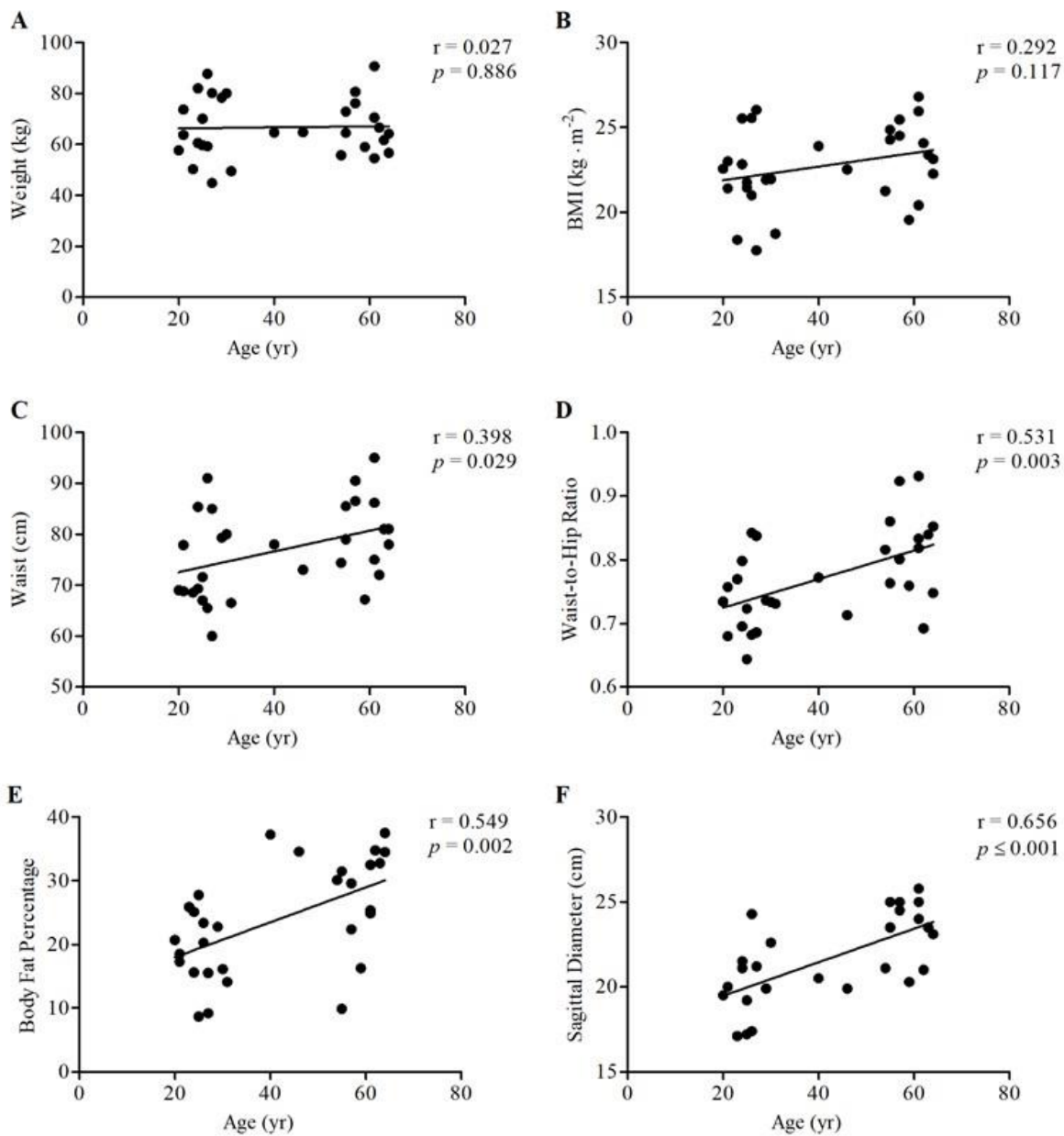
Figure 1. The associations of age with anthropometric characteristics in middle-aged and young adults. These data suggest age, independent of weight gain or changes in BMI (panels A and B), is associated with the increased accumulation of centrally located visceral adiposity, identified by increased waist circumference, W:H ratio, body fat percentage, and sagittal diameter (panels C-F).
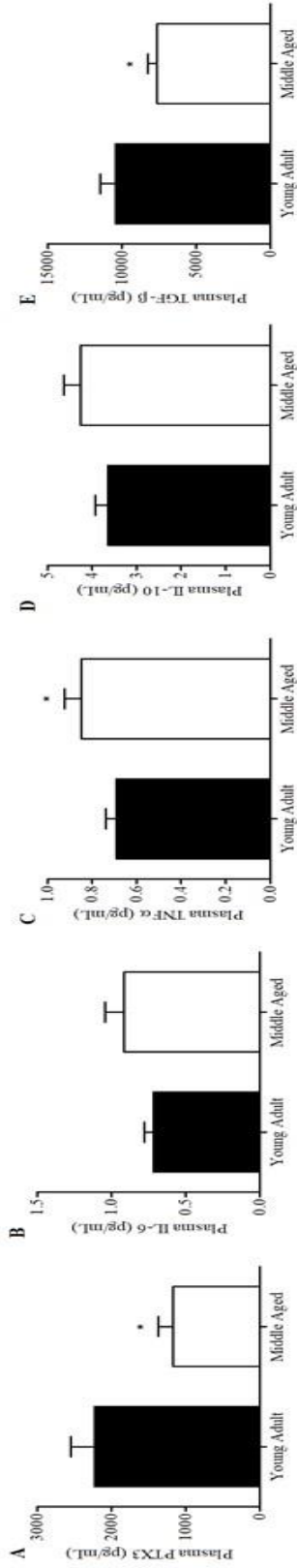
Figure 2. Plasma PTX3 and senescent-associated inflammatory cytokine concentrations. Plasma PTX3 concentrations were significantly lower in middle-aged compared to young adults (panel A). In addition, no differences in the pro-inflammatory cytokine IL-6 were observed (panel B), whereas plasma TNF-α concentration were greater in middle-aged compared to young adults (panel C). Likewise, no differences in the anti-inflammatory cytokine IL-10 were observed (panel D), whereas plasma TGF-β concentrations were lower in middle-aged compared to young adults (panel E). * indicates a significant difference in middle-aged compared to young adults ($p \leq 0.05$).

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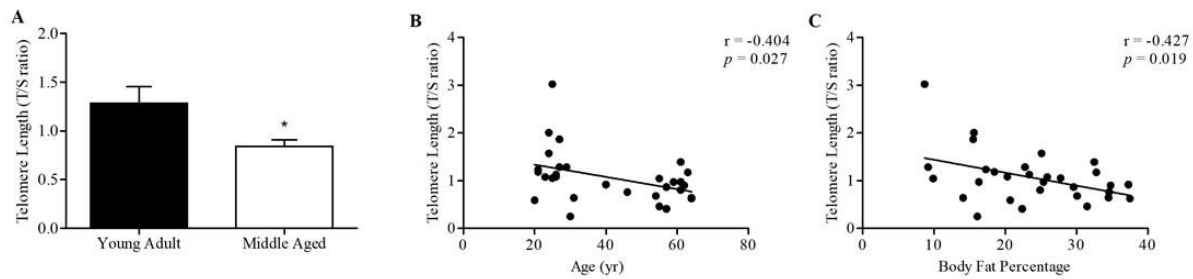
Figure 3. Relative telomere lengths (T/S ratio) and the associations with age and body fat percentage. Telomere lengths measured from isolated from PBMCs were significantly shorter in middle-aged compared to young adults (panel A). In addition, telomere lengths were negatively associated with increased age and body fat percentage (panels B and C). * indicates a significant difference in middle-aged compared to young adults ($p \leq 0.05$).
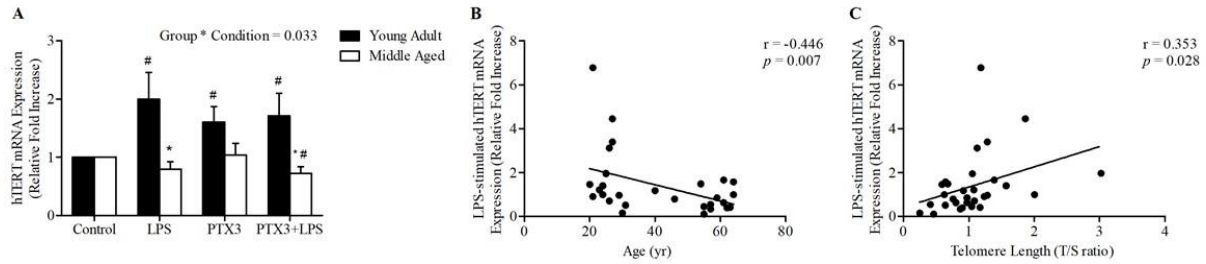
Figure 4. Expression of hTERT mRNA following 4-hour stimulation of isolated PBMCs with LPS and the associations with age and relative telomere length. hTERT mRNA expression was impaired in middle-aged and young adults following ex vivo stimulation of isolated PBMCs with LPS, PTX3, and PTX3+LPS (panel A). LPS-stimulated hTERT mRNA expression was negatively associated with age (panel B) and positively associated with relative telomere lengths (T/S ratio (panel C). * indicates a significant difference in middle-aged compared to young adults; # indicates a significant difference compared to unstimulated control culture conditions ($p \leq 0.05$).
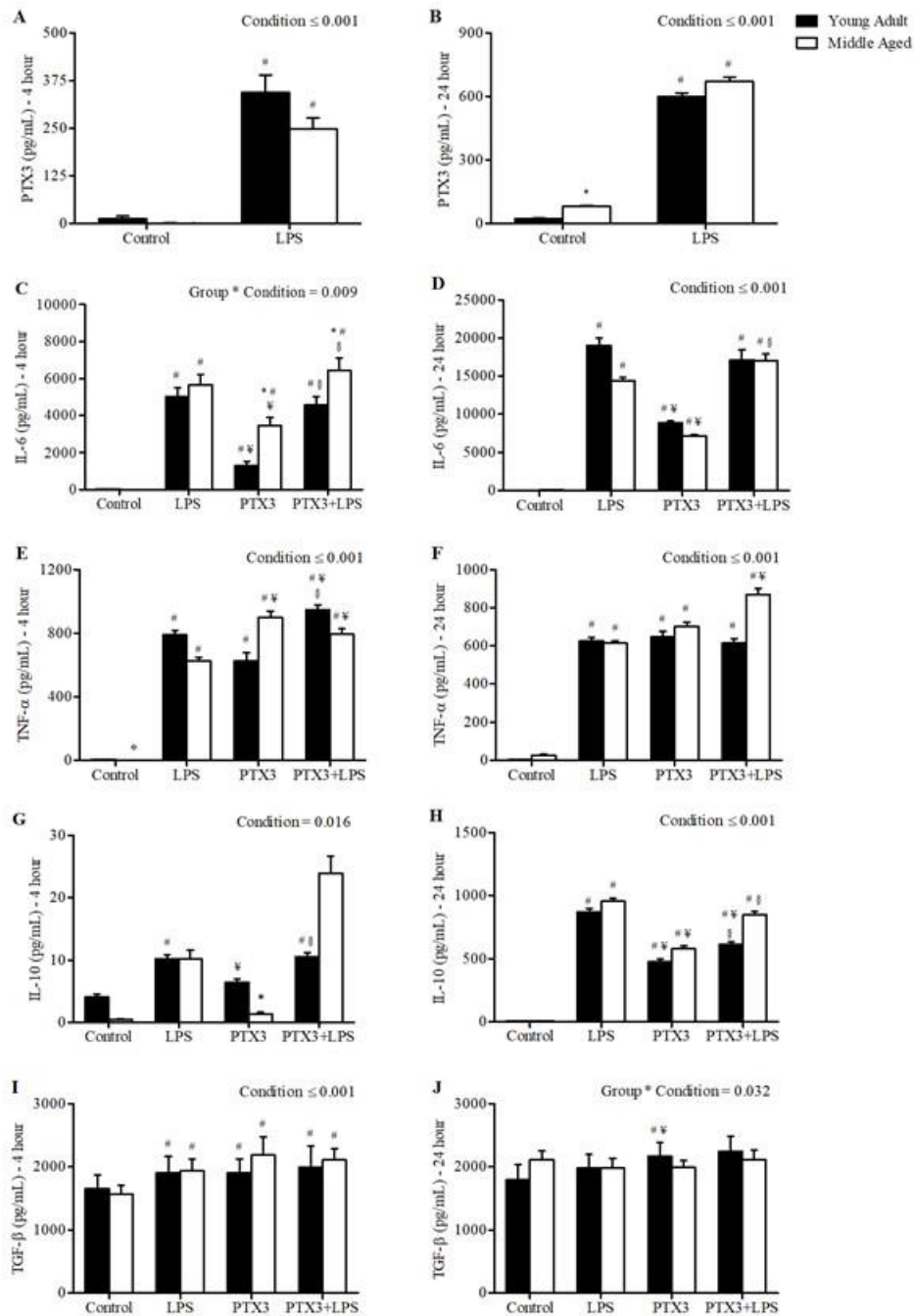
Figure 5. *Ex vivo* production of LPS-stimulated PTX3 (panels A and B) and the senescent-associated inflammatory cytokines following 4- and 24-hour stimulation of isolated PBMCs with LPS, PTX3, and PTX3+LPS in middle-aged and young adults (panels C-J). In addition, the capacity of PTX3 to produce and alter the LPS-stimulated production of senescent-associated

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inflammatory cytokines *ex vivo* was examined. * indicates a significant difference in middle-aged compared to young adults; # indicates a significant difference compared to unstimulated control culture conditions; ¥ indicates a significant difference compared to LPS-stimulated culture conditions; § indicates a significant difference compared to PTX3-stimulated culture conditions ($p \leq 0.05$).

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CHAPTER 6

DISCUSSION

Increasingly more evidence suggests that elevated plasma PTX3 concentrations serve as a biomarker of the severity of pro-inflammatory disease. While plasma PTX3 concentrations observed under pathological conditions have been shown to reach $\geq$ 100 ng/mL (Muller et al., 2001; Mairuhu et al., 2005; Sprong et al., 2009), these values are about 50- to 200-fold higher compared to those measured in healthy, young populations ($\sim$ 0.5 – 2 ng/mL) (Slusher et al., 2015; 2016a; 2016b). In fact, resting plasma PTX3 concentrations appear to be decreased in otherwise healthy obese and/or middle-aged populations (Osorio-Conles et al., 2011; Slusher et al., 2015). The role regular participation in physical activity exerts on mediating plasma PTX3 concentrations has yet to be fully elucidated. For example, Miyaki et al. (2011; 2012a; 2014) have routinely demonstrated that resting plasma PTX3 concentrations are elevated in physically active compared to inactive males, and in physically active adults who were previously inactive. Likewise, elevated plasma PTX3 concentrations are highly correlated with improved cardiovascular and metabolic health. However, results obtained from our laboratory suggest that resting plasma PTX3 concentrations are not significantly altered with increased aerobic fitness. It is important to note that a variety of variables may have potentially influenced these conflicting results, including environmental and genetic influences. Therefore, future studies should consider examining the potential influence of regular participation in physical activity across the lifespan on plasma PTX3 concentrations to better understand the utilization of PTX3 as a circulating biomarker of innate immune function and overall immunological health. Additional areas of potential research should also consider the identification of the major cellular source (e.g., neutrophils, adipose tissues, and skeletal muscle) of plasma PTX3 concentrations at rest and in response to physical activity to further identify potential target that may help restore a key component of the inflammatory milieu.

Our laboratory has hypothesized that increased exposure of circulating immune cells to elevated concentrations of PTX3 in plasma may help facilitate an anti-inflammatory phenotype prior to their internalization within inflamed or damaged tissue (Slusher et al., 2016b). Consequently, the anti-inflammatory phenotype of these immune cells may also help counterregulate localized inflammation linked to the progression of cardiovascular or metabolic disease pathology. While our results support the capacity of PTX3 to facilitate the production of anti-inflammatory cytokines (e.g., IL-10 and TGF-β1), PTX3 does not appear to downregulate the pro-inflammatory response of PBMCs following stimulation with LPS or the saturated fatty acid palmitate under the cell culture conditions examined (*Manuscripts 1 and 4*). These results are in conflict with those previously presented by Shiraki et al. (2016), suggesting that differences in our results may be potentially due to the utilization of human cells freshly isolated from circulation compared to immune cell lines utilized by Shiraki et al. (2016). Nonetheless, additional focus on manipulations of PTX3 concentrations, pre-incubation strategies prior to LPS/palmitate stimulation, and cell specific responses (e.g., differences within specific monocyte subsets and/or monocyte-differentiated macrophage phenotype), may help further elucidate the capacity of PTX3 to mediate the cellular production of inflammatory proteins. Likewise, increased manipulations of cellular receptors and intracellular signaling pathways that activate a wider spectrum of transcription factors, and thus the production of a larger array of inflammatory proteins, would potentially help elucidate how PTX3 functions within the pro- and anti-inflammatory milieu present in individuals with various levels of health and physical fitness across the lifespan.

Finally, our results highlight that hTERT, a central mechanism involved in the maintenance of telomere lengths, is impaired within immune cells of middle-aged compared to young adults. Furthermore, this age-related cellular dysregulation occurs prior to the onset of cell senescence

and may contribute to the shortening of telomere lengths often observed during middle-age. These findings are the first to demonstrate in humans the potential consequences of inflammatory-induced cellular replication previously described in basic cell line research. However, dysregulation of telomeric-associated mechanisms extends well beyond hTERT, and recent advances in telomeric measurement techniques (e.g., Flow-FISH) and cell sequencing methodologies (e.g., RNA-sequencing) have enabled researchers to investigate the relationship of telomere lengths with full transcriptomic variations within specific immune cell subpopulations and their corresponding subsets. Moreover, a recent study by Wang et al. (2017) have simultaneously isolated DNA and RNA from the same cell to examine the relationship of telomere length with the genomic profiles. While such methodological approaches have yet to be examined within humans, the emphasis on understanding the molecular underpinnings that may attenuate age-related pathology remains the next frontier of cellular physiology.

REFERENCES

1. Abbasi A, Fehrenbach E, Hauth M, et al. Changes in spontaneous and LPS-induced *ex vivo* cytokine production and mRNA expression in male and female athletes following prolonged exhaustive exercise. *Exerc. Immunol. Rev*. 2013; 19: 8-28.

2. Abderrahim-Ferkoune A, Bezy O, Chiellini C, et al. Characterization of the long pentraxin PTX3 as a TNFα-induced secreted protein of adipose cells. *J. Lipid Res.* 2003; 44: 994-1000.

3. Aderka D, Le JM, Vilcek J. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. *J. Immunol.* 1989; 143: 3517-3523.

4. Akiyama M, Hideshima T, Hayashi T, et al. Cytokines modulate telomerase activity in a human multiple myeloma cell line. *Cancer Res.* 2002; 62: 3876-3882.

5. Albright JM, Dunn RC, Shults JA, Boe DM, Afshar M, Kovacs EJ. Advanced age alters monocyte and macrophage responses. *Antioxid. Redox Signal.* 2016; 25: 805-815.

6. Alles VV, Bottazzi B, Peri G, Golay J, Introna M, Mantovani A. Inducible expression of PTX3, a new member of the pentraxin family, in human mononuclear phagocytes. *Blood* 1994; 84: 3483-3493.

7. Alonso-Fernández P, Puerto M, Maté I, Ribera JM, de la Fuente M. Neutrophils of centenarians show function levels similar to those of young adults. *J. Am. Geriatr. Soc.* 2008; 56: 2244-2251.

8. Alvarez-Rodríguez L, López-Hoyos M, Muñoz-Cacho P, Martínez-Taboada VM. Aging is associated with circulating cytokine dysregulation. *Cell Immunol.* 2012;273(2):124-132.

9. American College of Sports Medicine. (2013). *ACSM's guidelines for exercise testing and prescription*. Lippincott Williams & Wilkins.

10. Arem H, Moore SC, Patel A, et al. Leisure time physical activity and mortality: A detailed pooled analysis of the dose-response relationship. *JAMA Intern. Med.* 2015; 175: 959-967.

11. Baker DJ, Wijshake T, Tchkonia T, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 2011; 479: 232-236.

12. Belge K-U, Dayyani F, Horelt A, et al. The proinflammatory CD14+ CD16+ DR++ monocytes are a major source of TNF. *J. Immunol.* 2002; 168: 3536-3542.

13. Benetos A, Okuda K, Lajemi M, et al. Telomere length as an indicator of biological aging the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension* 2001; 37: 381-385.

14. Blackburn EH, Gall JG. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in Tetrahymena. *J. Mol. Biol.* 1978; 120: 33-53.

15. Booth S, Florida-James GD, McFarlin BK, Spielmann G, O'Connor DP, Simpson RJ. The impact of acute strenuous exercise on TLR2, TLR4 and HLA.DR expression on human blood monocytes induced by autologous serum. *Eur. J. Appl. Physiol*. 2010; 110:1259-1268.

16. Bories G, Caiazzo R, Derudas B, et al. Impaired alternative macrophage differentiation of peripheral blood mononuclear cells from obese subjects. *Diab. Vasc. Dis. Res.* 2012; 9: 189-195.

17. Bottazzi B, Inforzato A, Messa M, et al. The pentraxins PTX3 and SAP in innate immunity, regulation of inflammation and tissue remodelling. *J. Hepatol.* 2016; 64: 1416–1427.

18. Bozza S, Bistoni F, Gaziano R, et al. Pentraxin 3 protects from MCMV infection and reactivation through TLR sensing pathways leading to IRF3 activation. *Blood* 2006; 108: 3387-3396.

19. Bozza S, Campo S, Arseni B, et al. PTX3 binds MD-2 and promotes TRIF-dependent immune protection in aspergillosis. *J. Immunol.* 2014; 193: 2340-2348.

20. Breviario F, d'Aniello EM, Golay J, et al. Interleukin-1-inducible genes in endothelial cells. *J. Biol. Chem.* 1992; 267: 22190–22197.

21. Cartier A, Côté M, Lemieux I, et al. Age-related differences in inflammatory markers in men: contribution of visceral adiposity. *Metabolism* 2009; 58: 1452-1458.

22. Cawthon R. Telomere measurement by quntitative PCR. *Nucleaic Acids Res.* 2002; 30: e47.

23. Chazaud B, Brigitte M, Yacoub-Youssef H, et al. Dual and beneficial roles of macrophages during skeletal muscle regeneration. *Exerc. Sport Sci. Rev*. 2009; 37: 18-22.

24. Chu SH, Park J-H, Lee MK, et al. The association between pentraxin 3 and insulin resistance in obese children at baseline and after physical activity intervention. *Clinica. Chimica. Acta.* 2012; 413: 1430-1437.

25. Coppé JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol.* 2008; 6: 2853-2868.

26. Craig CL, Marshall AL, Sjöström M, et al. International physical activity questionnaire: 12-Country reliability and validity. *Med. Sci. Sports Exerc.* 2003; 35: 1381–1395.

27. Cros J, Cagnard N, Woollard K, et al. Human CD14 dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*. 2010; 33: 375-386

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28. Cuevas MJ, Almar M, García-Glez JC, et al. Changes in oxidative stress markers and NF-κB activation induced by sprint exercise. *Free Radic. Res*. 2005; 39: 431-439.

29. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, et al. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003; 426: 194-198.

30. D'Angelo C, De Luca A, Zelante T, et al. Exogenous pentraxin 3 restores antifungal resistance and restrains inflammation in murine chronic granulomatous disease. *J. Immunol.* 2009; 183: 4609-4618.

31. Da-Silva N, Arasaradnam R, Getliffe K, Sung E, Oo Y, Nwokolo C. Altered mRNA expression of telomere binding proteins (TPP1, PTT1, RAP1, TRF1 and TRF2) in ulcerative colitis and Crohn's disease. *Dig. Liver Dis.* 2010; 42: 544-548.

32. De Martinis M, Franceschi C, Monti D, Ginaldi L. Inflamm-ageing and lifelong antigenic load as major determinants of ageing rate and longevity. *FEBS Lett.* 2005; 579:2035-2039.

33. Deban L, Russo RC, Sironi M, et al. Regulation of leukocyte recruitment by the long pentraxin PTX3. *Nat. Immunol.* 2010; 11: 328–334.

34. Dias AA, Goodman AR, Dos Santos JL, et al. TSG-14 transgenic mice have improved survival to endotoxemia and to CLP-induced sepsis. *J. Leukoc. Biol.* 2001; 69: 928-936.

35. Dimitrov S, Hulteng E, Hong S. Inflammation and exercise: Inhibition of monocytic intracellular TNF production by acute exercise via β_2-adrenergic activation. *Brain Behav. Immun*. 2017; 61: 60-68.

36. Doni A, Mantovani G, Porta C, et al. Cell-specific regulation of PTX3 by glucocorticoid hormones in hematopoietic and nonhematopoietic cells. *J. Biol. Chem.* 2008; 283: 29983-29992.

37. Doni A, Michela M, Bottazzi B, et al. Regulation of PTX3, a key component of humoral innate immunity in human dendritic cells: stimulation by IL-10 and inhibition by IFN-γ. *J. Leukoc. Biol.* 2006; 79: 797-802.

38. Dubravec DB, Spriggs DR, Mannick JA, Rodrick ML. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. U. S. A*. 1990; 87: 6758-6761.

39. Durrer C, Francois M, Neudorf H, Little JP. Acute high-intensity interval exercise reduces human monocyte toll-like receptor 2 expression in type 2 diabetes. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2017; 312: R529-538.

40. Ershler WB. Interleukin-6: A Cytokine for Gerontolgists. *J. Am. Geriatr. Soc.* 1993; 41: 176-181.

41. Escobar-Morreale H, Martínez-García M, Montes-Nieto R, Fernández-Durán E, Temprano-Carazo S, Luque-Ramírez M. Effects of glucose ingestion on circulating inflammatory mediators: Influence of sex and weight excess. *Clin. Nutr.* 2017; 36: 522–529.

42. Fattori E, Cappelletti M, Costa P, et al. Defective inflammatory response in interleukin 6-deficient mice. *J. Exp. Med*. 1994; 180: 1243-1250.

43. Febbraio MA, Starkie RL. The cellular origin of plasma cytokine expression after acute exercise. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2002; 282: R1253-1257.

44. Fernando MR, Reyes JL, Iannuzzi J, Leung G, McKay DM. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. *PLoS One*. 2014; 9: e94188.

45. Franceschi C, Bonafè M, Valensin S, et al. Inflamm-aging: an evolutionary perspective on immunosenescence. *Ann. NY. Acad. Sci.* 2000a; 908:244-254.

46. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J. Gerontol. A. Biol. Sci. Med. Sci.* 2014; S1: S4-9.

47. Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood*. 1996; 87: 373-377.

48. Frenck RW, Blackburn EH, Shannon KM. The rate of telomere sequence loss in human leukocytes varies with age. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95: 5607-5610.

49. Gizard F, Heywood EB, Findeisen HM, et al. Telomerase activation in atherosclerosis and induction of telomerase reverse transcriptase expression by inflammatory stimuli in macrophages. *Arterioscler. Thromb. Vasc. Biol.* 2011; 31: 245-52.

50. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat. Rev. Immunol*. 2011; 11: 607-615.

51. Greider CW, Blackburn EH. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* 1989; 337: 331-337.

52. Guha M, Mackman N. The PI3K-Akt pathway limits LPS activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.* 2002; 277: 32124-32132.

53. Han X, Kitamoto S, Wang H, Boisvert WA. Interleukin-10 overexpression in macrophages suppresses atherosclerosis in hyperlipidemic mice. *FASEB J.* 2010; 24: 2869-2880.

54. Hayflick L, Moorhead PS, The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 1961; 25: 585-621.

55. Hearps AC, Martin GE, Angelovich TA, et al. Aging is associated with chronic innate immune activation and dysregulation of monocyte phenotype and function. *Aging Cell.* 2012; 11: 867-875.

56. Heidenreich PA, Trogdon JG, Khavjou OA, et al. Forecasting the future of cardiovascular disease in the United States: A policy statement from the American Heart Association. *Circulation* 2011; 123: 933-944.

57. Hermann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* 2001; 107: 67-77.

58. Hong S, Mills PJ. Effects of an exercise challenge on mobilization and surface marker expression of monocyte subsets in individuals with normal vs. elevated blood pressure. *Brain Behav. Immun*. 2008; 22: 590-599.

59. Huang C-J, Webb HE, Beasley KN, McAlpine DA, Tangsilsat SE, Acevedo EO. Cardiorespiratory fitness does not alter plasma pentraxin 3 and cortisol reactivity to acute psychological stress and exercise. *Appl. Physiol. Nutr. Metab.* 2014; 39: 375–380.

60. Imamura M, Kawasaki T, Savchenko AS, et al. Lipopolysaccharide induced expression of pentraxin 3 in human neutrophils and monocyte-derived macrophages. *Cell. Immunol.* 2007; 248: 86-94.

61. Introna M, Alles VV, Castellano M, et al. Cloning of mouse ptx3, a new member of the pentraxin gene family expressed at extrahepatic sites. *Blood* 1996; 87: 1862-1872.

62. Iwama H, Ohyashiki K, Ohyashiki JH, et al. Telomeric length and telomerase activity vary with age in peripheral blood cells obtained from normal individuals. *Hum. Genet.* 1998; 102: 397-402.

63. Jaillon S, Peri G, Delneste Y, et al. The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. *J. Exp. Med.* 2007; 204: 793–804.

64. Jenny NS, Arnold AM, Kuller LH, Tracy RP, Psaty BM. Associations of pentraxin 3 with cardiovascular disease and all-cause death: The cardiovascular health study. Arterioscler. *Thromb. Vasc. Biol.* 2009; 29: 594–599.

65. Jurk D, Wilson C, Passos JR, et al. Chronic inflammation induces telomere dysfunction and accelerate ageing in mice. *Nat. Commun.* 2014; 2: 4172.

66. Kahn S, Hull R, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444: 840–846.

67. Kawai T, Adachi O, Ogawa T, Takeda K. Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* 1999; 11: 115-122.

68. Kim D, Kim JY. Anti-CD14 antibody reduces LPS responsiveness via TLR4 internalization in human monocytes. *Mol. Immunol*. 2014; 57: 210-215

69. Kleemann R, Zadelaar S, Kooistra T. Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovasc. Res.* 2008; 79: 360-376.

70. Kohara K. Sacropenic obesity in aging population: current status and future directions for research. *Endocrine* 2014; 45: 15-25.

71. Kuk KL, Saunders TJ, Davidson LE, Ross R. Age-related changes in total and regional fat distribution. *Ageing Res. Rev.* 2009; 8: 339-348.

72. Kvist H, Chowdhury B, Grangård U, Tylén U, Sjöström L. Total and visceral adipose-tissue volumes derived from measurements with computed tomography in adult men and women: predictive equations. *Am. J. Clin. Nutr.* 1988; 48: 1351-1361.

73. Kyo S, Kanaya T, Takakura M, Ito H, Namiki M, Inoue M. hTERT is a critical determinant of telomerase activity in renal-cell carcinoma. *Int. J. Cancer.* 1998; 78: 539-543.

74. Lancaster GI, Khan Q, Drysdale P, et al. The physiological regulation of Toll-like receptor expression and function in humans. *J. Physiol*. 2005; 563: 945-955.

75. Lee GW, Goodman AR, Lee TH, Vilcek J. Relationship of TSG-14 protein to the pentraxin family of major acute phase proteins. *J. Immunol.* 1994; 153: 3700-3707.

76. Lee GW, Lee TH, Vilček J.TSG-14, a tumor necrosis factor- and IL-1-inducible protein, is a novel member of the pentraxin family of acute phase proteins. *J. Immunol.* 1993; 150: 1804-1812.

77. Lee JY, Ye J, Gao Z, et al. Reciprocal modulation of TLR4-signaling pathways involving MyD88 and PI3K/AKT by saturated and polyunsaturated fatty acids. *J. Biol. Chem.* 2003; 278: 37041-37051.

78. Lee MS, Choi S-E, Ha ES, et al. Fibroblast growth factor-21 protects human skeletal muscle myotubes from palmitate-induced insulin resistance by inhibiting stress kinase and NF-Kb. *Metabolism* 2012; 61: 1142-1151.

79. Lee TH, Lee GH, Ziff E, Vilček J. Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts. *Mol. Cell. Biol.* 1990; 5: 1982-1988.

80. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002; 105: 1135-1143.

81. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. M*ethods.* 2001; 25: 402-408.

82. Loprinizi PD. Cardiorespiratory capacity and leukocyte telomere length among adults in the United States. *Am. J. Epidemiol.* 2015; 182: 198-201.

83. Loprinzi PD, Loenneke JP, Blackburn EH. Movement-based behaviors and leukocyte telomere length among US adults. *Med. Sci. Sports Exerc.* 2015; 47: 2347-52.

84. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systemic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; 380: 2095-2128.

85. Lumeng CN, Liu J, Geletka L, et al. Aging is associated with an increase in T cells and inflammatory macrophages in visceral adipose tissue. *J. Immunol*. 2011; 187: 6208-6216.

105

86.   Maina V, Cotena A, Doni A, et al. Coregulation in human leukocytes of the long pentraxin PTX3 and TSG-6. *J. Leukoc. Biol.* 2009; 86: 123-132.

87.   Mairuhu AT, Peri G, Setiati TE, et al. Elevated plasma levels of the long pentraxin, pentraxin 3, in severe dengue virus infections. *J. Med. Virol.* 2005; 76: 547-552.

88.   Merino A, Buendia P, Martin-Male A, Aljama P, Ramirez R, Carracedo J. Senescent CD14$^+$CD16$^+$ monocytes exhibit proinflammatory and proatherosclerotic activity. *J. Immunol.* 2011; 186: 1809-1815.

89.   Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, Nojima A, Nabetani A, Oike Y, Matsubara H, Ishikawa F, Komuro I. A critical role for adipose tissue p53 in the regulation of insulin resistance. *Nat. Med.* 2009; 15: 1082-1087.

90.   Miyaki A, Choi Y, Maeda S. Pentraxin 3 Production in the Adipose Tissue and the Skeletal Muscle in Diabetic-Obese Mice. *Am. J. Med. Sci.* 2014; 347: 228–233.

91.   Miyaki A, Maeda S, Choi Y, et al. Association of plasma pentraxin 3 with arterial stiffness in overweight and obese individuals. *Am. J. Hypertens.* 2013; 26: 1250-1255.

92.   Miyaki A, Maeda S, Choi Y, Akazawa N, Tanabe Y, Ajisaka R. Habitual aerobic exercise increases plasma pentraxin 3 levels in middle-aged and elderly women. *Appl. Physiol. Nutr. Metab.* 2012a; 37: 907-911.

93.   Miyaki A, Maeda S, Choi Y, et al. The addition of whole-body vibration to a lifestyle modification on arterial stiffness in overweight and obese women. *Artery Res.* 2012b; 6: 85-91.

94.   Miyaki A, Maeda S, Otsuki T, Ajisaka R. Plasma pentraxin3 concentration increases in endurance-trained men. *Med. Sci. Sports Exerc.* 2011; 43: 12-17.

95.   Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell* 2011; 145: 341-355.

96.   Morrisette-Thomas V, Cohen AA, Fülöp T, et al. Inflamm-aging does not simply reflect increases in pro-inflammatory markers. *Mech Ageing Dev.* 2014; 139: 49-57.

97.   Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, *et al.* A highly conserved repetitive DNA sequence,(TTAGGG) n, present at the telomeres of human chromosomes. *Proce. Natl. Acad. Sci. U. S. A.* 1988; 85: 6622-6626.

98.   Mukherjee R, Barman PK, Thatoi PK, Tripathy R, Das BK, Ravindran B. Non-classical monocytes display inflammatory features: validation in sepsis and systemic lupus erythematous. *Sci. Rep.* 2015; 5: 13886.

99.   Muller B, Peri G, Doni A, et al. Circulating levels of the long pentraxin PTX3 correlated with severity of infection in critically ill patients. *Crit. Care Med.* 2001; 29: 1404-1407.

100.   Nakajima T, Kurano M, Hasegawa T, et al. Pentraxin3 and high-sensitive C-reactive protein are independent inflammatory markers released during high-intensity exercise. *Eur. J. Appl. Physiol.* 2010; 110: 905–913.

101. Nelson G, Wordsworth J, Wang C, Jurk D, Lawless C, Martin-Ruiz C, von Zglinicki T. A senecent cell bystander effect: senescence-induced senescence. *Aging Cell* 2012; 11: 345-349

102. Norata GD, Marchesi P, Pulakazhi Venu VK, et al. Deficiency of the long pentraxin ptx3 promotes vascular inflammation and atherosclerosis. *Circulation* 2009; 120: 699–708.

103. O'Donovan A, Pantell MS, Puterman E, et al. Cumulative inflammatory load is associated with short leukocyte telomere length in the Health, Aging and Body Composition Study. *PLoS One.* 2011; 6: e19687.

104. Oeppen J, Vaupel JW. Broken limits to life expectancy. *Science.* 2002; 296: 1029-1031.

105. Ogawa T, Kawano Y, Imamura T, et al. Reciprocal contribution of pentraxin 3 and C-reactive protein to obesity and metabolic syndrome. *Obesity* 2010; 18: 1871-1874.

106. Oliveira M, Gleeson M. The influence of prolonged cycling on monocyte Toll-like receptor 2 and 4 expression in healthy men. *Eur. J. Appl. Physiol.* 2010; 109:251-257.

107. Osorio-Conles O, Guitart M, Chacón M, et al. Plasma PTX3 protein levels inversely correlate with insulin secretion and obesity, whereas visceral adipose tissue PTX3 gene expression is increased in obesity. *Am. J. Physiol. Endocrinol. Metab.* 2011; 301: E1254-E1261.

108. Pålsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology.* 2004; 113: 153-162.

109. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood.* 1989; 74: 2527-2534.

110. Pavanello S, Stendardo M, Mastrangelo G, et al. Inflammatory long pentraxin 3 is associated with leukocyte telomere length in night-shift workers. *Front. Immunol.* 2017; 8: 516.

111. Petersen A, Pedersen B. The role of IL-6 in mediating the anti inflammatory. *J. Physiol. Pharmacol.* 2006; 57: 43-51

112. Pinheiro da Silva FP, Aloulou M, Benhamou M, Monteiro RC. Inhibitory ITAMs: a matter of life and death. *Trends Immunol.* 2008; 29: 366-373.

113. Pinheiro da Silva FP, Aloulou M, Skurnik D, et al. CD16 promotes Escherichia coli sepsis through an FcRγ inhibitory pathway that prevents phagocytosis and facilitates inflammation. *Nat. Med.* 2007; 13: 1368-1374.

114. Polentarutti N, Bottazzi B, Di Santo E, et al. Inducible expression of the long pentraxin PTX3 in the central nervous system. *J. Neuroimmunol.* 2000; 106: 87-94.

115. Pols TW, Nomura M, Harach T, et al. TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. *Cell Metab.* 2011; 14: 747-757.

116. Pucci S, Fisco T, Zonetti MJ, Bonanno E, Mazzarelli P, Mauriello A. PTX3: A modulator of human coronary plaque vulnerability acting by macrophages type 2. *Int. J. Cardiol.* 2014; 176: 710-717.

107

117. Radom-Aizik S, Zaldivar FP, Haddad F, Cooper DM. Impact of brief exercise on circulating monocyte gene and microRNA expression: implications for atherosclerotic vascular disease. *Brain Behav. Immun*. 2014; 39: 121-129.

118. Ramunas J, Yakubov E, Brady JJ, et al. Transient delivery of modified mRNA encoding TERT rapidly extends telomeres in human cells. *FASEB J.* 2015; 29: 1930-1939.

119. Redon S, Reichenback P, Lingner J. The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Res.* 2010; 38: 5797-5806.

120. Riley JC. Rising life expectancy: a global history. Cambridge University Press, 2001.

121. Rolph MS, Zimmer S, Bottazzi B, Garlanda C, Mantovani A, Hansson GK. Production of the Long Pentraxin PTX3 in Advanced Atherosclerotic Plaques. *Arter. Thromb. Vasc. Biol.* 2012; 22: 10–14.

122. Röth A, Yssel H, Pène J, et al. Telomerase levels control the lifespan of human T lymphocytes. *Blood* 2003; 102: 849-857.

123. Salio M, Chimenti S, De Angelis N et al. Cardioprotective function of the long pentraxin PTX3 in acute myocardial infarction. *Circulation* 2008; 117: 1055-1064.

124. Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 2010; 10: 170-181.

125. Schmidt JC, Dalby AB, Cech TR. Identification of human TERT elements necessary for telomerase recruitment to telomeres. *eLife* 2014; 1: 3.

126. Selkirk GA, McLellan TM, Wright HE, Rhind SG. Mild endotoxemia, NF-κB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2008; 295: R611-23.

127. Shalova IN, Kajiji T, Lim JY, et al. CD16 regulates TRIF-dependent TLR4 response in human monocytes and their subsets. *J. Immunol*. 2012; 188: 3584-3593.

128. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid–induced insulin resistance. *J. Clinical Invest.* 2006; 116: 3015-3025.

129. Shiraki A, Kotooka N, Komoda H, Hirase T, Oyama J-I, Node K. Pentraxin-3 regulates the inflammatory activity of macrophages. *Biochem. Biophys. Rep.* 2016; 5: 290-295.

130. Simpson RJ, McFarlin BK, McSporran C, Spielmann G, ó Hartaigh B, Guy K. Toll-like receptor expression on classic and pro-inflammatory blood monocytes after acute exercise in humans. *Brain Behav. Immun*. 2009; 23: 232-239.

131. Sindhu S, Al-Roub A, Koshy M, Thomas R, Ahmad R. Palmitate-induced MMP-9 expression in the human monocytic cells is mediated through the TLR4-MyD88 dependent mechanism. *Cell Physiol. Biochem.* 2016; 39: 889-900.

132. Slusher AL, Huang C-J. Association of pentraxin 3 with insulin resistance and glucose response following maximal aerobic exercise in obese and normal-weight individuals. *Can. J. Physiol. Pharmacol.* 2016a; 94: 734-738.

133.   Slusher AL, Huang C-J, Acevedo EO. The potential role of aerobic exercise-induced Pentraxin 3 on obesity-related inflammation and metabolic dysregulation. *Med. Inflamm.* 2017b; 2017: 1092738.

134.   Slusher AL, Mischo AB, Acevedo EO. Pentraxin 3 is an anti-inflammatory protein associated with lipid-induced interleukin 10 in vitro. *Cytokine* 2016b; 86: 36–40.

135.   Slusher AL, Mock JT, Whitehurst M, Maharaj A, Huang C-J. The impact of obesity on pentraxin 3 and inflammatory milieu to acute aerobic exercise. *Metabolism* 2015; 64: 323-329.

136.   Slusher AL, Shibata Y, Whitehurst M, Maharaj A, Quiles JM, Huang CJ. Exercise reduced pentraxin 3 levels produced by endotoxin-stimulated human peripheral blood mononucelar cells in obese individuals. *Exp. Biol. Med*. 2017a; 242: 1279-1286.

137.   Sprong T, Peri G, Neeleman C, et al. Pentraxin 3 and C-reative protein in severe meningococcal disease. *Shock* 2009; 31: 28-32.

138.   Spyridopoulos I, Martin-Ruiz C, Hilkens C, et al. CMV seropositivity and T-cell senescence predict increases cardiovascular mortality in octogenarians: results from the Newcastle 85+ study. *Aging Cell* 2016; 15: 389-392.

139.   Starkie R, Ostrowski SR, Jauffred S, Febbraio M, Pedersen BK. Exercise and IL-6 infusion inhibit endotoxin-induced TNF-α production in humans. *FASEB J*. 2003; 17: 884-886.

140.   Starr ME, Evers BM, Saito H. Age-associated increase in cytokine production during systemic inflammation: adipose tissue as a major source of IL-6. *J. Gerontol. A. Biol. Sci. Med. Sci.* 2009; 64: 723-730.

141.   Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am. J. Physiol. Endocrinol. Metab*. 2003; 285: E433-437

142.   Steppich B, Dayyani F, Gruber R, Lorenz R, Mack M, Ziegler-Heitbrock HL. Selective mobilization of CD14+ CD16+ monocytes by exercise. *Am. J. Physiol. Cell Physiol*. 2000; 279: C578-586.

143.   Stevens J, Cai J, Pamuk ER, et al. The effect of age on the association between body-mass index and mortality. *N. Engl. J. Med.* 1998; 338: 1-7.

144.   Sun H, Tian J, Xian W, Xie T, Yang X. Pentraxin-3 attenuates renal damage in diabetic nephropathy by promoting M2 macrophage differentiation. *Inflammation* 2015; 38: 1739-1747.

145.   Taniguchi T, Koido Y, Aiboshi J, Yamashita T, Suzaki S, Kurokawa A. Change in the ratio of interleukin-6 to interleukin-10 predicts a poor outcome in patients with systemic inflammatory response syndrome. *Crit. Care Med.* 1999; 27: 1262-1264.

146.   Tchkonia T, Morbeck DE, von Zglinkicki T, et al. Fat tissue, aging, and cellular senescence. *Aging Cell* 2010; 9: 667-684.

147. Timmerman KL, Flynn MG, Coen PM, Markofski MM, Pence BD. Exercise training-induced lowering of inflammatory (CD14+ CD16+) monocytes: a role in the anti-inflammatory influence of exercise? *J. Leukoc. Biol*. 2008; 84: 1271-1278.

148. Tzanetakou IP, Katsilambros NL, Benetos A, Mikhailidis DP, Perrea DN. "Is obesity linked to aging?": adipose tissue and the role of telomeres. *Aging Res. Rev.* 2012; 11: 220-229.

149. Van Gaal LF, Mertens IL, Christophe E. Mechanisms linking obesity with cardiovascular disease. *Nature*. 2006; 444: 875-880.

150. Vaure C, Liu Y. A comparative review of Toll-like receptor 4 expression and functionality in different animal species. *Front. Immunol*. 2014; 5: 316

151. Von der Thüsen JH, Kuiper J, Fekkes ML, de Vos P, van Berker JT, Biessen EA. Attenuation of atherogenesis by systemic and local adenovirus-mediated gene transfer of interleukin-10 in LDLr−/− mice. *FASEB J.* 2001; 15: 2730-2732.

152. Wang C, Zhao L, Lu S. Role of TERRA in the regulation of telomere length. *Int. J. Biol. Sci.* 2015; 11: 316-323.

153. Weiss EP, Reeds DN, Ezekiel UR, Albert SG, Villareal DT. Circulating cytokines as determinants of weight loss-induced improvements in insulin sensitivity. *Endocrine* 2017; 55: 153–164.

154. Witasp A, Carrero JJ, Michaëlsson K, et al. Inflammatory biomarker pentraxin 3 (PTX3) in relation to obesity, body fat depots and weight loss. *Obesity* 2014; 22: 1373-1379..

155. Wong KL, Tai JJ, Wong WC, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood*. 2011; 118: e16-31.

156. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*. 1990;49: 1431-1434.

157. Wu D, Ren Z, Pae M, et al. Aging up-regulates expression of inflammatory mediators in mouse adipose tissue. *J. Immunol.* 2007; 179: 4829-4839.

158. Yamamoto M, Sato S, Hemmi H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; 301: 640-643.

159. Yamasaki K, Kurimura M, Kasai T, Sagara M, Kodama T, Inoue K. Determination of physiological plasma pentraxin 3 (PTX3) levels in healthy populations. *Clin. Chem. Lab. Med.* 2009; 47: 471-477.

160. Yang HS, Woo JE, Lee SJ, Park SH, Woo JM. Elevated plasma pentraxin 3 levels are associated with development and progression of diabetic retinopathy in Korean patients with type 2 diabetes mellitus. *Invest. Ophthalmol. Vis. Sci.* 2014; 55: 5989-5997.

161. Yu G-L, Bradley JD, Attardi LD, Blackburn EH. In vivo alteration of telomere sequences and senescence caused by mutated Tetrahymena telomerase RNAs. *Nature* 1990; 344: 126-132.

162. Zamboni M, Rossi AP, Fantin F, et al. Adipose tissue, diet and aging. *Mech. Ageing Dev.* 2014; 136-137: 129-137.

163. Zempo-Miyaki A, Fujie S, Sato K. Elevated pentraxin 3 level at the early stage of exercise training is associated with reduction of arterial stiffness in middle-aged and older adults. *J. Hum. Hypertens.* 2016; 30: 521-526.

164. Zou Y, Sfeir A, Gryaznov SM, Shay JW, Wright WE. Does a sentinel or a subset of short telomeres determine replicative senescence? *Mol. Biol. Cell* 2004; 15: 3709-3718.

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PEER REVIEWED BOOK CHAPTERS

1. Huang C-J., McAllister M.J., **Slusher A.L.** Cardiovascular Health Implications: the Roles of Psychological stress, Physical Activity, and Dietary Modifications. In Oxford Research Encyclopedia of Psychology. *Oxford University Press: New York, NY – Published Online 2017* doi: 10.1093/acrefore/9780190236557.013.208.

2. **Slusher A.L.,** Acevedo E.O. Mental Health and Physical Activity. The Oxford Encyclopedia of Sport, Exercise, and Performance Psychology. *Oxford University Press: New York, NY – Accepted with Revisions*.

PEER REVIEWED SCIENTIFIC PUBLICATIONS

1. **Slusher A.L.,** Zúñiga T.M., Acevedo E.O. Maximal Exercise Alters the Inflammatory Phenotype of Mononuclear Cells and Response to *Ex Vivo* LPS Stimulation. *Medicine and Science in Sports and Exercise 2018 [Epub ahead of print].* doi:10.1249/MSS.0000000000001480.

2. Fico, B.G., Whitehurst, M., **Slusher A.L.,** Mock, J.T., Maharaj, A., Dodge, K.M., Huang, C-J. The Comparison of High-Intensity Interval Exercise versus Continuous Moderate-Intensity Exercise on Plasma Calprotectin and Associated Inflammatory Mediators. *Physiology and Behavior 2017;183:27-32.* doi:10.1016/j.physbeh.2017.10.015.

3. **Slusher A.L.,** Shibata Y., Whitehurst M., Maharaj A., Quiles J.M., Huang C-J. Exercise-Reduced Pentraxin 3 Levels Produced by Endotoxin-Stimulated Human Peripheral Blood Mononuclear Cells in Obese Individuals. *Experimental Biology and Medicine 2017;242:1279-1286.* doi: 10.1177/1535370217706963.

4. **Slusher A.L.,** Huang C-J., Acevedo E.O. The Potential Role of Aerobic Exercise-Induced Pentraxin 3 on Obesity-Related Inflammation and Metabolic Dysregulation. *Mediators of Inflammation 2017;2017:1092738.* doi: 10.1155/2017/1092738.

5. Acevedo E.O., **Slusher A.L.** Physiological Activation to Acute Mental Challenge: Implications of Cardiovascular Health. *Kinesiology Reviews 2017;6:120-133.* doi: 10.1111/bph.12325.

6. **Slusher A.L.,** Mischo A.B., Acevedo E.O. Pentraxin 3 is an Anti-Inflammatory Protein Associated with Lipid-Induced Interleukin 10 *In Vitro. Cytokine 2016;86:36-40.* doi: 10.1016/j.cyto.2016.07.012.

7. **Slusher A.L.,** Huang C-J. Association of Pentraxin 3 with Insulin Resistance and Glucose Response Following Maximal Aerobic Exercise in Obese and Normal-mass Individuals. *Canadian Journal of Physiology and Pharmacology 2016;94(7):734-738.* doi: 10.1139/cjpp-2015-0464.

8. Maharaj A., **Slusher A.L.,** Zourdos M.C., Whitehurst M., Fico B., Huang C-J. Association of Calprotectin with Leukocyte Chemotactic and Inflammatory Mediators Following Acute Aerobic Exercise. *Applied Physiology, Nutrition, and Metabolism 2016;41(1):83-87.* doi: 10.1139/apnm-2015-0385.

9. Huang C-J., **Slusher A.L.**, Whitehurst M., Wells M., Maharaj A., Shibata Y. The Impact of Acute Aerobic Exercise on Chitinase-3-like Protein 1 and Intelectin-1 Expression in Obesity. *Experimental Biology and Medicine 2016;241(2)216-221.* doi: 10.1177/1535370215602785.

10. Huang C-J., McAllister M., **Slusher A.L.,** Mock J.T., Webb H.E., Acevedo E.O. Obesity-Related Oxidative Stress: The Impact of Physical Activity and Diet Manipulation. *Sports Medicine – Open 2015;1:32.* doi: 10.1186/s40798-015-0031-y.

11. **Slusher A.L.,** Whitehurst M., Zoeller R.F., Mock J.T., Maharaj A., Huang C-J. Attenuated Fibroblast Growth Factor 21 Response to Acute Aerobic Exercise in Obese Individuals. *Nutrition, Metabolism, and Cardiovascular Disease 2015;25:839-845.* doi: 10.1016/j.numecd.2015.06.002.

12. **Slusher A.L.,** McAllister M., Huang C-J. A Therapeutic Role for Vitamin D on Obesity-Associated Inflammation and Weight-Loss Intervention. *Inflammation Research 2015;64:565-575.* doi: 10.1007/s00011-015-0847-4.

13. Huang C-J, **Slusher A.L,** Whitehurst M., Wells M., Mock J.T., Maharaj A., Shibata Y. Acute Aerobic Exercise Mediates G Protein-Coupled Receptor Kinase 2 Expression in Human PBMCs. *Life Sciences 2015;135:87-91.* doi: 10.1016/j.lfs.2015.05.018.

14. **Slusher A.L.,** Whitehurst M., Zoeller R.F., Mock J.T., Maharaj A., Huang C-J. Brain-Derived Neurotrophic Factor and Substrate Utilization Following Acute Aerobic Exercise in Obese Individuals. *Journal of Neuroendocrinology 2015;27:370-376.* doi: 10.1111/jne.12275.

15. Huang C-J., Stewart J.K., Shibata Y., **Slusher A.L.,** Acevedo E.O. Lipopolysaccharide-Binding Protein and Leptin are Associated with Stress-Induced Interleukin-6 Cytokine Expression *ex vivo* in Obesity. *Psychophysiology 2015;52:687-694.* doi: 10.1111/psyp.12387.

16. **Slusher A.L.,** Mock J.T., Whitehurst M., Maharaj A., Huang C-J. The Impact of Obesity on Pentraxin 3 and Inflammatory Milieu to Acute Aerobic Exercise. *Metabolism 2015;64:323-329.* doi: 10.1016/j.metabol.2014.10.022.

17. Huang C-J., Mari D. C., Whitehurst M., **Slusher A.L.,** Wilson A., Shibata Y. Brain-Derived Neurotrophic Factor Expression *ex vivo* in Obesity. *Psychology & Behavior 2014;123:76-79.* doi: 10.1016/j.physbeh.2013.10.004.

SCIENTIFIC MANUSCRIPTS UNDER REVIEW

1. Bao F., **Slusher A.L.,** Whitehurst M, Huang C-J. Circulating miRNAs are Upregulated in Response to Acute Aerobic Exercise in Obesity. *Frontiers in Physiology (In Review).*

2. **Slusher A.L.,** Whitehurst M., Maharaj A., Dodge, K.M., Fico, B.G., Mock J.T., Huang C-J. Plasma Pentraxin 3 and Glucose Kinetics to Acute High-Intensity Interval Exercise versus

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Continuous Moderate-Intensity Exercise. *Applied Physiology, Nutrition, and Metabolism (In Review).*

3. **Slusher A.L.** Zúñiga T.M., Acevedo E.O. Aerobic Fitness Alters the Capacity of Mononuclear Cells to Produce Pentraxin 3 Following Maximal Exercise. *European Journal of Applied Physiology (In Review).*

4. **Slusher A.L.,** Patterson V.T., Schwartz C.S., Acevedo E.O. Impact of High Intensity Interval Exercise on Executive Function and Brain-Derived Neurotrophic Factor in Healthy College Ages Males. *Physiology and Behavior (In Review).*

SCIENTIFIC MANUSCRIPTS IN PROGRESS

1. **Slusher A.L.,** Zúñiga T.M., Acevedo E.O. Influence of Aerobic Training Status on Fatty Acid-Induced hTERT mRNA Expression Following Maximal Exercise.

2. Dodge K.M., **Slusher A.L.,** Fico B.G., Garten R.S., Ferrandi P.J., Rodriguez A.A., Pena G., Huang C-J. The Impact of Obesity on Plasma Pentraxin 3 and Endothelial Function Following Acute High-Intensity Interval Exercise.

3. **Slusher A.L.,** Zúñiga T.M., Acevedo E.O. Stimulation of Isolated Mononuclear Cells with the Saturated Fatty Acid Palmitate Reduces hTERT mRNA Expression in Physically Active and Inactive Males.

4. Patterson V.T., **Slusher A.L.,** Schwartz C.S., Acevedo E.O. Perceptual Responses and Physiological Activation with Acute High Intensity Interval Ergometry in Sedentary Males.

5. Zúñiga T.M., **Slusher A.L.,** Mischo A.B., Acevedo E.O. Relationship of LPS-Induced iNOS mRNA Expression and the Pro-Apoptotic Signaling Pathway in Leukocytes of Healthy Trained Males.

6. Maharaj, A., Huang C-J., **Slusher A.L.,** Zourdos M.C., Pena, G., Whitehurst M. The Effects of Maximal Aerobic Exercise on Plasma BDNF and BDNF Expression in PBMCs in Obese and Non-Obese Subjects.

SCIENTIFIC PRESENTATIONS – ORAL

1. **Slusher A.L.,** Mischo A.B., Zúñiga T.M., Acevedo E.O. Maximal Exercise Alters the Inflammatory Phenotype of Mononuclear Cells and Response to *Ex Vivo* LPS Stimulation. *§ *** 2017 ACSM Annual Meeting (Denver, CO) –__Immunology Section__ – Med. Sci. Sports Exerc. 2017;49(5S):198.*

2. **Slusher A.L.,** Mischo A.B., Zúñiga T.M., Acevedo E.O. How Does Acute Aerobic Exercise Alter Innate Immune Function? **** 2017 SEACSM Annual Meeting (Greenville, SC) –__Pre-Conference Rapid Research Race__*

SCIENTIFIC PRESENTATIONS – POSTER

3. **Slusher A.L.,** Zúñiga T.M., Acevedo E.O. Influence of Aerobic Training Status on the Pentraxin 3 (PTX3)-Mediated Innate Immune Response Following Acute Exercise.
    * 2018 ACSM Annual Meeting (Minneapolis, MN)
    * 2018 SEACSM Annual Meeting (Chattanooga, TN)

4. Zúñiga T.M., **Slusher A.L.,** Acevedo E.O. Influence of Aerobic Training Status on Fatty Acid-Induced hTERT mRNA Expression Following Maximal Exercise.
    * 2018 ACSM Annual Meeting (Minneapolis, MN)
    * 2018 SEACSM Annual Meeting (Chattanooga, TN)

5. Schwartz C.S., **Slusher A.L.,** Patterson V.T., Acevedo E.O. Impact of High Intensity Interval Exercise on Executive Function and Brain Derived Neurotrophic Factor in Healthy Males.
    * 2018 ACSM Annual Meeting (Minneapolis, MN)
    * 2018 SEACSM Annual Meeting (Chattanooga, TN)

6. Dodge K.M., **Slusher A.L.,** Fico B.G., Garten R.S., Ferrandi P.J., Rodriguez A.A., Pena G., Huang C-J. The Impact of Obesity on Plasma Pentraxin 3 and Endothelial Function Following Acute High-Intensity Interval Exercise.
    * 2018 ACSM Annual Meeting (Minneapolis, MN)
    * 2018 SEACSM Annual Meeting (Chattanooga, TN)

7. Patterson V.T., **Slusher A.L.,** Acevedo E.O. Differences in Plasma and Serum BDNF in Response to Acute HIIE. American College of Sports Medicine Annual Meeting (2017), Denver, CO.
    § ** 2017 ACSM Annual Meeting (Denver, CO) – <u>High Intensity Exercise in Blood Marker Responses</u> – Med. Sci. Sports Exerc. 2017;49(5S):491.
    * 2017 SEACSM Annual Meeting (Greenville, SC) – <u>Doctoral Poster Research Award Nominee</u>

8. **Slusher A.L.,** Mischo A.B., Zúñiga T.M., Acevedo E.O. Maximal Exercise Alters the Inflammatory Phenotype of Mononuclear Cells and Response to *Ex Vivo* LPS Stimulation
    * 2017 SEACSM Annual Meeting (Greenville, SC) – <u>Doctoral Poster Research Award Nominee</u>

9. Huang C-J., **Slusher A.L.,** Ferrandi P.J., Whitehurst M. Apoptosis of Human Peripheral Blood Mononuclear Cells Following Maximal Aerobic Exercise in Obesity.
    § * 2017 ACSM Annual Meeting (Denver, CO) – Med. Sci. Sports Exerc. 2016;49(5S):458.

10. Pena, G., Maharaj, A., Huang C-J., Zourdos M.C., **Slusher A.L.,** Whitehurst M. The Effects of Maximal Aerobic Exercise on Plasma BDNF and BDNF Expression in PBMCs in Obese and Non-Obese Subjects.
    § * 2017 ACSM Annual Meeting (Denver, CO) – Med. Sci. Sports Exerc. 2016;49(5S):700.
    * 2017 SEACSM Annual Meeting (Greenville, SC)

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11. Fico B.G., Whitehurst M., **Slusher A.L.,** Mock J.T., Maharaj A., Dodge K., Huang C-J. The Comparison of High-Intensity Interval Exercise vs. Continuous Moderate-Intensity Exercise-Mediated Calprotectin and Inflammatory Mediators.
*§ * 2017 ACSM Annual Meeting (Denver, CO) – Med. Sci. Sports Exerc. 2016;49(4S):491.*
* 2017 SEACSM Annual Meeting (Greenville, SC)
* 2016 Florida Atlantic University College of Education 16th Student Achievement Council Annual Research Symposium (Boca Raton, FL)

12. Bao F., Whitehurst M., **Slusher A.L.,** Maharaj A., Mock J.T., Huang C-J. Inflammatory-Associated Circulating MicroRNAs are not Regulated in Response to Acute High-Intensity Interval Exercise in Healthy Males.
*§ * 2017 ACSM Annual Meeting (Denver, CO) – Med. Sci. Sports Exerc. 2016;49(5S):491.*
* 2017 SEACSM Annual Meeting (Greenville, SC)

13. Dodge K.M., Whitehurst M., **Slusher A.L.,** Fico B.G., Maharaj A., Mock J.T., Huang C-J. Pentraxin 3 and Glucose Kinetic Responses to Acute High-Intensity Interval Exercise vs. Continuous Moderate-Intensity Exercise.
*§ * 2017 ACSM Annual Meeting (Denver, CO) – Med. Sci. Sports Exerc. 2016;48(5S):274.*
* 2017 SEACSM Annual Meeting (Greenville, SC)
* 2016 Florida Atlantic University College of Education 16th Student Achievement Council Annual Research Symposium (Boca Raton, FL)

14. Ferrandi P.J., **Slusher A.L.,** Whitehurst M., Huang C-J. The Expression of BAX and BCL-2 in Peripheral Blood Mononuclear Cells Following Maximal Exercise in Obese and Normal-Weight Individuals.
* 2017 SEACSM Annual Meeting (Greenville, SC)

15. **Slusher A.L.,** Shibata Y., Whitehurst M., Maharaj A., Quiles J.M., Huang C-J. Exercise-Mediated Pentraxin 3 Expression from *In Vitro* Stimulation of Human PBMCs with Lipopolysaccharide in Obese Individuals.
*§ * 2016 ACSM Annual Meeting (Boston, MA) – Med. Sci. Sports Exerc. 2016; 48(5S): 743.*
* 2016 SEACSM Annual Meeting (Greenville, SC)

16. Patterson V.T., **Slusher A.L.,** Caslin H.L., Schwartz C.S., Acevedo E.O. High Intensity Interval Ergometry: Power Output, Catecholamines, Substance P, and Perceived Pain.
*§ * 2016 ACSM Annual Meeting (Boston, MA) – Med. Sci. Sports Exerc. 2016;48(5S):115.*

17. Bao F., **Slusher A.L.,** Whitehurst M., Huang C-J. Circulating MicroRNAs are Upregulated in Response to Acute Aerobic Exercise in Obesity.
*§ * 2016 ACSM Annual Meeting (Boston, MA) – Med. Sci. Sports Exerc. 2016;48(5S):742.*
* 2016 SEACSM Annual Meeting (Greenville, SC) – <u>Master Poster Research Award Nomine</u>

18. Maharaj A., **Slusher A.L.,** Zourdos M., Whitehurst M., Fico B.G., Huang C-J. Association of Calprotectin with Leukocyte Chemotactic and Inflammatory Mediators Following Acute Aerobic Exercise.
*§ * 2016 ACSM Annual Meeting (Boston, MA) – Med. Sci. Sports Exerc. 2016;4(5S):86.*

* 2016 SEACSM Annual Meeting (Greenville, SC)

19. Fico B.G., **Slusher A.L.,** Whitehurst M., Maharaj A., Huang C-J. The Impact of Obesity on Calprotectin Response to Acute Aerobic Exercise.
   § * 2016 ACSM Annual Meeting (*Boston, MA*) *– Med. Sci. Sports Exerc. 2016;48(5S):743.*
   * 2016 SEACSM Annual Meeting (Greenville, SC)
   ** 2015 Florida Atlantic University College of Education 15th Student Achievement Council Annual Research Symposium (Boca Raton, FL) – **Health and Medical Sciences Category SECOND place***

20. Patterson V.T., **Slusher A.L.,** Acevedo E.O. High Intensity Interval Ergometry, Substance P, and Cognitive Function: An Exploratory Study.
   * 2016 SEACSM Annual Meeting (Greenville, SC)

21. Ferrandi P.J., Whitehurst M., **Slusher A.L.,** Pena, G.S., Maharaj A., Mock J.T., Huang C-J. The Expression of BAX and BCL-2 in Peripheral Blood Mononuclear Cells Following Maximal Exercise in Obese and Normal-Weight Individuals.
   * 2016 Florida Atlantic University College of Education 16th Student Achievement Council Annual Research Symposium (Boca Raton, FL)

22. Bao F., Whitehurst M., **Slusher A.L.,** Maharaj A., Mock J.T., Huang C-J. Inflammatory-Associated Circulating MicroRNAs are not Regulated in Response to Acute High-Intensity Interval Exercise in Healthy Males.
   * 2016 Florida Atlantic University College of Education 16th Student Achievement Council Annual Research Symposium (Boca Raton, FL)

23. Huang C-J., Stewart J.K., Shibata Y., **Slusher A.L.,** Acevedo E.O. Lipopolysaccharide-Binding Protein and Leptin are Associated with Stress-Induced Interleukin-6 Cytokine Expression *ex vivo* in Obesity.
   § * 2015 ACSM Annual Meeting (San Diego, CA) – Med. Sci. Sports Exerc. 2015;47(5S):893.

24. **Slusher A.L.,** Mock J.T., Whitehurst M., Maharaj A., Huang C-J. The Impact of Obesity on Pentraxin 3 and Inflammatory Milieu to Acute Aerobic Exercise.
   § * 2015 ACSM Annual Meeting (San Diego, CA) – Med. Sci. Sports Exerc. 2015;47(5S):235.
   ** 2015 SEACSM Annual Meeting (Jacksonville, FL) – **Master Poster Research Award SECOND Place***

25. Maharaj A., **Slusher A.L.**, Whitehurst M., Zoeller R.F., Mock J.T., Huang C-J. Fibroblast Growth Factor 21 Expression and Insulin Resistance to Acute Aerobic Exercise in Obese Individuals.
   § * 2015 ACSM Annual Meeting (San Diego, CA) – Med. Sci. Sports Exerc. 2015;47(5S):236.
   ** 2015 SEACSM Annual Meeting (Jacksonville, FL) – **Undergraduate Poster Research Award FIRST Place***

26. Maharaj A., Urtula A.M., **Slusher A.L.,** Mock J.T., Fisher C.B., Huang C-J. Pentraxin 3 Reactivity to Acute Exercise in Obese Individuals.

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* 2014 Florida Atlantic University College of Education 14th Student Achievement Council Annual Research Symposium (Boca Raton, FL)

27. **Slusher A.L.,** Mock J.T., Whitehurst M., Zoeller R.F., Maharaj A., Fisher C.B., Huang C-J. Relationship Between Brain-Derived Neurotrophic Factor and Substrate Utilization in Healthy Individuals.
§ * 2014 ACSM Annual Meeting (Orlando, FL) – Med. Sci. Sports Exerc. 2014;46(5S):677-678.
* 2014 SEACSM Annual Meeting (Greenville, SC)
* 2013 Florida Atlantic University College of Education 13th Student Achievement Council Annual Research Symposium (Boca Raton, FL)

§ Published Abstract
* Poster Presentation
** Thematic Poster Presentation
*** Oral Presentation

GRANT SUBMISSIONS

Principle Investigator, "High Intensity Interval Exercise as a Therapeutic Approach to Reverse Immune Cell Aging in Obese Individuals"
Program: Banting Postdoctoral Fellowship (Dr. Eli Puterman, Faculty Mentor, University of British Columbia)
Sponsor: Canadian Institutes of Health Research
Appl # RN325922
June 1, 2018 – May 30, 2020
($150,000 CAD – Unfunded)

Co-Investigator, "Impact of 24 weeks of aerobic exercise on cellular changes associated with telomere biology and immunosenescence: A randomized controlled trial"
Program: Project Grant (PI: Dr. Eli Puterman, University of British Columbia)
Sponsor: Canadian Institutes of Health Research
Appl # RN339362 - 391817
January 2018 - December 2020
($285,000 CAD – Unfunded)

Co-Investigator, "Promoting biological stress adaptation and immune health through habitual physical activity in nurses: A randomized controlled trial."
Program: Project Grant (PI: Dr. Eli Puterman, University of British Columbia)
Sponsor: Canadian Institutes of Health Research
Appl # RN338780 - 390932
January 2018 - December 2020
($1,085,000 CAD – Unfunded)
Co-Investigator, "Exercise Induced Changes in Telomere Biology"
Heart and Stroke Foundation, Canada
July 1, 2018 – June 30, 2020
($179,543 CAD – Unfunded)

Principle Investigator, "High Intensity Interval Exercise as a Therapeutic Approach to Reverse Immune Cell Aging in Obese Individuals"
Program: Izaak Walton Killam Postdoctoral Research Fellowship (Dr. Eli Puterman, Faculty Mentor, University of British Columbia)
Sponsor: University of British Columbia, Vancouver
July 1, 2018 – June 31, 2020
(**$104,000** – Unfunded)

Principle Investigator, "Counterregulatory Effects of Pentraxin 3 on Obesity Related Pro-Inflammation and Cellular Aging."
Program: Foundation Doctoral Student Research Grant (Dr. Edmund O. Acevedo, Faculty Mentor, Virginia Commonwealth University)
Sponsor: American College of Sports Medicine
July 1, 2017 – June 31, 2018
(**$5,000** – Unfunded)

COMMUNITY PRESENTATIONS

"DESSERTS" is Stressed Spelled Backwards: Discuss Overcoming Stress through Mindfulness and Physical Activity. Richmond Public Library (November, 2017)

UNIVERSITY SERVICE

2017   **Panel Member – "Surviving and Flourishing in Graduate School"**
College of Humanities and Sciences: New Graduate Teaching Assistant Welcome
Virginia Commonwealth University

2006-2008   **Student Athlete Advisory Committee**
Wilmington College

2007   **Dean of Students Search Committee Student Representative**
Wilmington College

2005   **Wilmington College Incoming Student Leadership Council**
Wilmington College

CERTIFICATIONS

2010   **National Strength and Conditioning Association Certified Strength & Conditioning Specialist**
Certification Number: 201070912

PROFESSIONAL AFFILIATIONS

Since 2014   The International Society of Exercise Immunology
Since 2012   American College of Sports Medicine
Since 2012   Southeast Region American College of Sports Medicine

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INVITED MANUSCRIPT REVIEWER

Acta Physiologica
Journal of Neuroimmunology
Saudi Medical Journal
International Journal of Preventive Medicine
Molecular and Cellular Endocrinology
Journal of Neuroendocrinology
International Journal for Vitamin and Nutritional Research
International Journal of Sport Nutrition & Exercise Metabolism
Obesity-Open Access
Surgery for Obesity and Related Diseases