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Age-related arterial immune cell infiltration in mice is attenuated by caloric restriction or voluntary exercise

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Abstract

Age-related arterial inflammation is associated with dysfunction of the arteries and increased risk for cardiovascular disease. To determine if aging increases arterial immune cell infiltration as well as the populations of immune cells principally involved, we tested the hypothesis that large elastic and resistance arteries in old mice would exhibit increased immune cell infiltration compared to young controls. Additionally, we hypothesized that vasoprotective lifestyle interventions such as life-long caloric restriction or 8 weeks of voluntary wheel running would attenuate age-related arterial immune cell infiltration. The aorta and mesenteric vasculature with surrounding perivascular adipose was excised from young normal chow (YNC, 4–6 months, n = 10), old normal chow (ONC, 28–29 months, n = 11), old caloric restricted (OCR, 28–29 months, n = 9) and old voluntary running (OVR, 28–29 months, n = 5) mice and digested to a single cell suspension. The cells were then labeled with antibodies against CD45 (total leukocytes), CD3 (pan T cells), CD4 (T helper cells), CD8 (cytotoxic T cells), CD19 (B cells) CD11b and F4/80 (macrophages) and analyzed by flow cytometry. Total leukocytes, T cells (both $CD4^+$ and $CD8^+$ subsets), B cells and macrophages in both aorta and mesentery were all 5–6 fold greater in ONC compared to YNC. Age-related increases in T cell (both $CD4^+$ and $CD8^+$), B cell and macrophage infiltration in aorta were abolished in OCR mice. OVR mice exhibited 50% lower aortic T cell and normalized macrophage infiltration. B cell infiltration was not effected by VR. Age-related mesenteric $CD8^+$ T cell and macrophage infiltration was normalized in OCR and OVR mice compared to young mice, whereas B cell infiltration was normalized by CR but not VR. Splenic $CD4^+$ T cells from ONC mice exhibited a 3 fold increase in gene expression for the T helper (Th)1 transcription factor, Tbet, and a 4 fold increase in FoxP3, a T regulatory cell transcription factor,

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compared to YNC. Splenic B cells and mesenteric macrophages from old mice exhibited decreased proinflammatory cytokine gene expression regardless of treatment group. These results demonstrate that aging is associated with infiltration of immune cells around both the large-elastic and resistance arteries and that the vasoprotective lifestyle interventions, CR and VR, can ameliorate age-related arterial immune cell infiltration.

Keywords

inflammation; T cells; B cells; macrophages; aorta; mesentery

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the industrialized world and aging is the primary and best predictive risk factor for future CVD diagnosis (Lakatta 2003). A majority of CVDs are diseases of the arteries including myocardial infarction, stroke, and peripheral artery disease. Vascular aging contributes to increased risk for CVD primarily through increases in large-artery stiffness and impairments in endothelium-dependent dilation (EDD) (Blackwell *et al.,* 2004; Donato *et al.,* 2007; Gerhard *et al.,* 1996; Lakatta and Levy 2003). Associated with age-related arterial dysfunction is arterial inflammation which has been observed in both humans and animals (Belmin *et al.,* 1995; Donato *et al.,* 2008; Donato *et al.,* 2007; Morgan *et al.,* 2013; Song *et al.,* 2012). Further, inhibition of inflammatory signaling can improve arterial function in older adults (Pierce *et al.,* 2009; Walker *et al.,* 2012). In the characterization of age-related arterial inflammation, the vast majority of investigations have focused on inflammatory signals originating from the blood vessel *per se.*

In the past decade, evidence has emerged that cells from both the innate and adaptive immune systems directly contribute to arterial dysfunction induced by acute experimental hypertension. Specifically, these investigations have found that T cells, B cells and macrophages accumulate in the perivascular tissue that surrounds large arteries and that these immune cells induce vascular dysfunction (Chan *et al.,* 2015; Guzik *et al.,* 2007; Wenzel *et al.,* 2011). More recently, $CD8^+$ T cells have been shown to be the T cell subset primarily responsible for increased arterial stiffness and impaired EDD associated with experimental hypertension (Trott *et al.,* 2014; Wu *et al.,* 2014). Whether a similar phenomenon occurs with advancing age and which immune cell subtypes might contribute to age-related arterial inflammation is unknown.

Certain lifestyle interventions can improve arterial function in older humans and rodents. Specifically, both our laboratory and others have contributed to extensive evidence demonstrating that caloric restriction (CR) (Csiszar *et al.,* 2009; Donato *et al.,* 2013; Fornieri *et al.,* 1999; Lynch *et al.,* 1999; Rippe *et al.,* 2010; Walker *et al.,* 2014) as well as exercise (DeSouza *et al.,* 2000; Durrant *et al.,* 2009; Fleenor *et al.,* 2010; Jablonski *et al.,* 2015; Lesniewski *et al.,* 2011; Trott *et al.,* 2009) improve vascular function in large elastic and resistance arteries in old rodents and humans while also reducing markers of arterial inflammation. Using a histological approach, our laboratory has found increases in total T

cells and macrophages in aortas from old mice and that voluntary running (VR) reduces macrophage infiltration in old mice (Lesniewski *et al.,* 2011). However, such histological staining was semi-quantitative and only allowed for the analysis of a small section of artery. In this investigation, we employed flow cytometry to assess infiltration of T cells (both $CD4^+$ and $CD8^+$ subtypes), B cells and macrophages in the whole aorta (large elastic artery) and mesenteric vascular arcade (resistance arteries) with age. We chose both artery types as large elastic arteries play an important role in dampening pulse pressure whereas resistance arteries regulate systemic vascular resistance and local tissue perfusion. Further, we sought to determine whether CR or VR would influence arterial immune cell infiltration and immune cell gene expression in old mice.

For the present study, we hypothesized that aging would result in increased infiltration of both $CD4^+$ and $CD8^+$ T cells, B cells and macrophages in both the aorta and mesenteric vascular arcade. We also hypothesized that CR or VR would attenuate age-related immune cell infiltration. Lastly, we hypothesized that aging would promote proinflammatory immune cell gene expression and that this would be ameliorated by CR or VR.

2. MATERIALS AND METHODS

2.1 Mice

All animal experiments conformed to the Guide and Use of Laboratory Animals and were approved by the University of Utah and Veteran's Affairs Medical Center-Salt Lake City (VAMC-SLC) Animal Care and Use Committees. Young male (4–6 month) B6D2F1 mice were obtained from Charles River Inc. and old (28–29 months) male B6D2F1 mice were obtained from the National Institute of Aging (NIA) colony maintained by Charles River Inc. All mice were housed in standard mouse cages on a 12:12 light:dark cycle in the animal facility at the VAMC-SLC. Four groups of mice were employed for this study: Young normal chow (YNC, n = 10), old normal chow (ONC, n = 11), old caloric restriction (OCR, n = 9) and old voluntary running (OVR, n =5). YNC, ONC and OVR mice were allowed access to normal mouse chow and water *ad libitum.* OCR mice were obtained from the NIA colony where caloric restriction is initiated at 14 weeks of age at approximately 10% below *ad libitum* intake, this is increased to approximately 25% restriction at 15 weeks and to approximately 40% restriction at 16 weeks, which is maintained throughout the life of the animal. ONC and OCR mice were randomized at the NIA colony at 14 weeks of age and singly housed. Out of all normal chow fed old mice, we randomized mice to either stay in their home cage (ONC) or to be singly housed in a cage containing a running wheel for eight weeks prior to sacrifice (OVR). Running distance for each mouse was monitored daily.

2.2 Flow cytometry and cell sorting

To remove circulating leukocytes from arteries, following sacrifice, the chest cavity was opened and the right atrium was nicked. A cannula was placed in the left ventricle and the animals were perfused at physiological pressure until the effluent was cleared of blood. Aortas were dissected from the aortic arch to the renal artery bifurcation, skeletal muscle and lymph nodes were removed but perivascular fat and connective tissue was left intact. Blood vessels and perivascular tissue of the mesenteric vascular arcade was dissected away

from the intestinal wall with care to avoid puncturing the wall and to avoid mesenteric lymph nodes. Perivascular tissue around both the aorta and mesenteric vascular arcade was included as this is where arterial immune cells accumulate (Guzik *et al.,* 2007; Lesniewski *et al.,* 2011).

Following dissection, spleens, aortas, and mesenteric vascular arcades were digested using collagenase type XI (125 U/ml), collagenase type I-S (450 U/ml), and hyaluronidase I-S (60 U/ml) dissolved in DPBS buffer containing calcium and magnesium for 30 min at 37°C. The tissues were further dispersed using repeated pipetting and the resultant homogenate was passed through a 70-μm sterile filter, yielding single-cell suspensions. Single cell suspensions were labeled with the following anti-mouse antibodies at a 1:100 concentration: Brilliant Violet-CD45 (total leukocytes), APC-CD3- (pan T cells), FITC-CD4 (T helper cells), PE-CD8 (cytotoxic T cells), APC Cy7-CD19 (B Cells), PE Cy7-CD11b and PerCP Cy5.5-F4/80 (macrophages). All antibodies were obtained from Tonbo Biosciences. Dead cells were labeled with Ghost Dye (Tonbo) and excluded from analysis. Cell subpopulations were assessed on a BD FACSAria and the following cell types were sorted into individual tubes: $CD4^+$ T cells, $CD8^+$ T cells, B cells and macrophages. The “flow minus one” technique was used to establish gating, as described previously (Trott *et al.,* 2014).

2.3 qPCR

Following sorting, total RNA from the cell samples was extracted using the RNeasy kit (QIAGEN) following manufacturer’s directions. In preliminary experiments, we found that T and B cells sorted from aorta and mesentery did not yield sufficient quantity and/or quality RNA so we elected to perform qPCR on splenic T and B cells. We used mesenteric macrophages as the mesentery contains a relatively large pool of tissue resident macrophages and because the spleen contains few fully differentiated macrophages. cDNA was synthesized using the Quantitect reverse transcription kit (QIAGEN). qPCR was performed on a BioRad CFX 96 Real Time system with RT^2 SYBR green master mix (QIAGEN) with primers for the following genes: *tnfa* fwd: CTGAACTTCGGGGTGATCGG, rev: GGCTTGTCACTCGAATTTTGAGA; *ifng* fwd: CCTGCGGCCTAGCTCTGA, rev: GCCATGAGGAAGAGCTGCA; *tbet* fwd: ACCAGAGCGGCAAGTGGG, rev: TGGACATATAAGCGGTTCCCTG; *gata3* fwd: CTACGCTCCTTGCTACTCAGG, rev: GGAGGGAGAGAGGAATCCGA; *prf1* fwd: TCTTGGTGGGACTTCAGCTTT, rev: TCCATACACCTGGCACGAAC *foxp3* fwd: GGCCCTTCTCCAGGACAGA, rev: GCTGATCATGGCTGGGTTGT; *il10* fwd: GCTCTTACTGACTGGCAT, rev: CGCAGCTCTAGGAGCATGTG *18s* fwd: TAGAGGGACAAGTGGCGTTC, rev: CGCTGAGCCAGTCAGTGT. 18s mRNA was used as the reference gene. Target gene expression fold change compared to YNC was determined using the ΔΔCt method.

2.4 Statistics

One way-ANOVA was used to determine group differences. Least squares differences *post hoc* tests were used when appropriate. ANCOVA was employed to determine whether arterial immune cell infiltration was influenced by body, visceral adipose or soleus muscle mass. Data are mean ± SEM. Means were considered significantly different at $p \leq 0.05$.

3. RESULTS

3.1 Animal and splenic leukocyte characteristics

Animal body mass, mesenteric vascular arcade, heart, soleus muscle and epididymal (visceral) fat pad weights are presented in Table 1. Visceral adipose tissue mass was smaller in ONC mice compared to YNC ($p < 0.05$, Table 1). The mass of the mesenteric vascular arcade and associated perivascular adipose followed a similar pattern (Table 1). Body, heart and spleen mass were smaller in OCR mice compared to ONC ($p < 0.05$, Table 1). Mice provided with voluntary running wheels (OVR) mice ran an average of 0.2 ± 0.04 km/day. Voluntary running normalized age-related decreases in soleus muscle and mesenteric vascular arcade mass ($p < 0.05$, Table 1).

We assessed the numbers and populations of splenic leukocytes to determine whether aging or aging combined with CR or VR alters the immune system on a systemic level. OCR mice exhibited a greater percentage of splenic monocytes compared to the other groups ($p < 0.05$, Table 2).

3.2 Arterial leukocyte infiltration

Both the aorta (Figure 1A) and mesentery (Figure 1B) of ONC mice exhibited 5-fold greater numbers of $CD45^+$ total leukocytes compared to YNC ($p < 0.05$). In the aorta, this increase was abolished in OCR mice and attenuated by 41% in OVR mice (Figure 1A, $p < 0.05$ vs. ONC). In the mesentery, CR attenuated ($p < 0.05$, −36%) and VR attenuated (−33%) mesenteric leukocyte infiltration although this was not significantly different from ONC ($p = 0.07$, Figure 1B). To control for differences in tissue mass, mesenteric leukocyte infiltration data is expressed as cell number per gram of tissue throughout. Importantly, ONC mice also had higher raw numbers of leukocytes per mesentery compared to YNC (78,781 ± 9,848 vs. 30,430 ± 3,399, $p < 0.05$) despite smaller tissue mass. Group differences in both aortic and mesenteric $CD45^+$ immune cell infiltration persisted when body mass, visceral adipose mass or soleus muscle mass were controlled for as covariates.

3.3 Arterial T cell infiltration

Aortas from ONC mice exhibited a 5-fold increase in $CD3^+$ T cell infiltration ($p < 0.05$ vs. YNC) that was abolished by CR and 50% lower in VR (Figure 2A, both $p < 0.05$ vs. ONC), the mesenteric vasculature exhibited a 50% lower $CD3^+$ immune cell infiltration in both OCR and OVR mice compared to ONC (Figure 2B, both $p < 0.05$). In the aorta, infiltration of both $CD4^+$ (T helper) and $CD8^+$ (T cytotoxic) subsets were attenuated by similar magnitudes to total T cells by either CR or VR (Figure 2C, both $p < 0.05$ vs. ONC. In the mesentery, both $CD4^+$ and $CD8^+$ subsets were increased 6-fold in ONC compared to YNC ($p < 0.05$). Caloric restriction and VR decreased $CD8^+$ T cells by 50% (both $p < 0.05$ vs. ONC) but had no effect on $CD4^+$ T cell infiltration (Figure 2D).

3.4 Arterial B cell infiltration

Aortas and mesenteries from ONC mice exhibited 6-fold greater B cell infiltration compared to YNC (Figure 3A & B, both $p < 0.05$). This increase was abolished in the aorta and decreased 50% in the mesentery by CR only ($p < 0.05$ vs. ONC).

3.5 Arterial macrophage infiltration

CD11b, F4/80 double positive macrophages were 4-fold greater in aorta and 2-fold greater in mesentery of ONC mice compared to YNC ($p < 0.05$, Figure 4A & B). Age-related increases in arterial macrophages were normalized by both CR and VR (Figure 4A & B, both $p < 0.05$ vs. ONC).

3.6 Immune cell gene expression

We examined gene expression of pro- and anti-inflammatory genes in splenic T and B cells and mesenteric infiltrating macrophages. In $CD4^+$ T helper (Th) cells, we found no gene expression differences for the proinflammatory cytokines tumor necrosis factor (TNF)-α (*tnfa*) and interferon (IFN)-γ (*ifng*) (Figure 5A). We examined Th cell transcription factors and found that the Th1 transcription factor, Tbet (*tbet),* was increased 3-fold in $CD4^+$ cells from ONC mice compared to YNC ($p < 0.05$), this increase was not altered in OCR or OVR mice (Figure 5A). In a similar manner, the T regulatory transcription factor, FoxP3 (*foxp3*), was increased 4-fold in $CD4^+$ cells from ONC mice compared to YNC ($p < 0.05$) and not normalized by CR or VR (Figure 5A). There were no differences in gene expression of the Th2 transcription factor, Gata3 (*gata3*) (Figure 5A). We found no differences in gene expression for *tnfa*, *ifng* and the cytotoxic protein perforin-1 (*prf1*) in $CD8^+$ (T cytotoxic) cells (data not shown). B cells exhibited no differences in *tnfa* gene expression; however, B cell *ifng* gene expression was decreased 50% in ONC, OCR and OVR compared to YNC mice ($p < 0.05$, Figure 5B). Although not statistically significant, ($p = 0.13$) B cells from ONC mice also exhibited greater anti-inflammatory cytokine Interleukin-10 (*il10*) gene expression compared to YNC (Figure 5B). Macrophages from ONC, OCR and OVR mice exhibited 50% lower *tnfa* gene expression compared to YNC ($p < 0.05$), *il10* expression was unchanged (Figure 5C).

4. DISCUSSION

4.1 Summary of major findings

The major findings of this investigation are as follows: 1) Aging is associated with significant increases in total leukocytes, T cells (both $CD4^+$ and $CD8^+$ subtypes), B cells and macrophages in both aorta and mesenteric vasculature and their surrounding perivascular tissue. 2) Age-related $CD4^+$ and $CD8^+$ T cell, B cell and macrophage infiltration in aorta and $CD8^+$ T cell, B cell and macrophage infiltration in mesentery is normalized by CR. 3) Voluntary running attenuates age-related $CD4^+$ and $CD8^+$ T cell and macrophage, but not B cell infiltration in aorta and also attenuates $CD8^+$ T cell and macrophage infiltration of the mesenteric vasculature. 4) Aging results in alterations in $CD4^+$ T cell transcription factor expression that is not normalized by CR or VR. 5) Aging results in decreased proinflammatory cytokine gene expression in B cells and macrophages that is not altered by CR or VR.

These results demonstrate that in addition to inflammatory signals emanating from the arterial wall, age-related arterial inflammation is also characterized by increased arterial infiltrating immune cells. In addition, these results demonstrate that vasoprotective lifestyle interventions result in decreased arterial immune cell burden. Lastly, aging is associated with

increased $CD4^+$ T cell proinflammatory transcription factor gene expression and decreased expression of proinflammatory cytokine gene expression in B cells and macrophages.

4.2 Aging, arterial inflammation and vascular function

The present investigation demonstrates increased $CD4^+$ and $CD8^+$ T cell, B cell and macrophage infiltration in the aorta and mesenteric vascular arcade with age. The concept that inflammation contributes to age-related arterial dysfunction is not new; however, the vast majority of investigations into age-related arterial inflammation focus on inflammatory mediators in the vascular smooth muscle and endothelium. (Belmin *et al.,* 1995; Donato *et al.,* 2008; Donato *et al.,* 2007; Morgan *et al.,* 2013; Song *et al.,* 2012; Spinetti *et al.,* 2004). The concept that immune cells *per se* contribute to arterial dysfunction was elucidated by an investigation by Guzik *et al.* where the authors found that T cells directly mediate arterial dysfunction in experimental hypertension (Guzik *et al.,* 2007). More recently, macrophages, B cells, and the subset of $CD8^+$ T cells specifically have been implicated in mediating arterial dysfunction using similar methods (Chan *et al.,* 2015; Trott *et al.,* 2014; Wenzel *et al.,* 2011; Wu *et al.,* 2014). In the present study we chose to focus on T cells, B cells and macrophages, as each has been shown to directly mediate arterial dysfunction. Wenzel et al. 2011, found that both macrophages and neutrophils infiltrated the aorta in experimental hypertension but that only macrophages directly induced endothelial dysfunction. Whether neutrophils or other innate immune cells infiltrate the vasculature with age and whether they play a role in age related arterial dysfunction is unknown and a topic for future study.

Our group was the first to report increased T cell and macrophage staining in the aortas of old (29–31 month) mice (Lesniewski *et al.,* 2011). Other groups have found increases in arterial immune cells in middle aged genetic models of cardiovascular disease (Wu *et al.,* 2015; Du *et al.,* 2016). Importantly, in the present study we employed a model of healthy aging, old (28–29 month) B6D2F1 mice. In a similar manner to our observations in arteries and associated perivascular adipose, aging has been shown to increase T cells and macrophages in visceral adipose tissue (Lumeng *et al.,* 2011). The mechanism of age-related arterial immune cell infiltration is unknown. There is evidence for arterial production of immune cell recruiting chemokines with age, whether these chemokines are produced by the artery *per se* or are adipokines originating from the surrounding perivascular adipose is as yet undetermined. Although we found the pattern of arterial immune cell infiltration was not dependent on changes in muscle or fat mass with age it is also possible that the sum production of adipo- and myokines might alter systemic immune cell migration and infiltration.

Whether immune cells directly contribute to age-related arterial dysfunction is unknown, however, there is evidence that cells from the aging immune system can contribute to arterial dysfunction in humans. Giant cell arteritis is an inflammatory disease of the arteries involving T cells and macrophages and the principal risk factor for development of this disease is advanced age (Weyand *et al.,* 2012). With age, T cells develop a proinflammatory, end differentiated T effector memory phenotype (Fagnoni *et al.,* 1996; Merino *et al.,* 1998; Weyand *et al.,* 2014). The proportion of end differentiated T effector memory cells are predictive of cardiovascular disease mortality in octogenarians and mortality after stroke

(Nadareishvili *et al.,* 2004; Spyridopoulos *et al.,* 2015). Further, patients with rheumatoid arthritis, have a greater proportion of these cells and are at greater risk for cardiovascular disease even when controlling for traditional risk factors (del Rincon *et al.,* 2001; Gerli *et al.,* 2004). Depletion of B cells in older adults with rheumatoid arthritis improves EDD (Hsue *et al.,* 2014). These data, combined with our observation of marked arterial immune cell infiltration support the concept that the immune system may be a contributor to age-related arterial dysfunction and risk for cardiovascular disease.

4.3 Vasoprotective lifestyle interventions and arterial immune cell infiltration

Data from this study demonstrate that the vasoprotective lifestyle interventions, CR and VR, can ameliorate age-related arterial immune cell infiltration. Caloric restriction increases lifespan in numerous mammalian species (Masoro 2005). Similar to previous observations (Chen *et al.,* 1998; Donato *et al.,* 2013), in this study, CR resulted in lower body, heart and spleen mass compared to old controls. Extensive evidence from our group and others show that CR promotes arterial health with age by preserving nitric oxide-bioavailability and EDD, preventing age-related large artery stiffening and, attenuating inflammatory signaling in the artery (Csiszar *et al.,* 2009; Donato *et al.,* 2013; Fornieri *et al.,* 1999; Lynch *et al.,* 1999; Rippe *et al.,* 2010; Walker *et al.,* 2014). This study is the first to demonstrate that CR also decreases arterial immune cell infiltration and suggests a decreased immune cell burden may contribute to preserved arterial function with CR. Notably, CR also normalizes age-related changes in the adaptive immune system in both rodents and non-human primates (Chen *et al.,* 1998; Messaoudi *et al.,* 2006) and decreases macrophage marker gene expression in visceral adipose of aged rats (Sierra Rojas *et al.,* 2016). Combined, this supports the concept that CR may have synergistic effects on both the artery and the immune system that act to preserve vascular function with age.

Exercise has also been shown to improve both EDD, large artery stiffness and decrease proinflammatory markers in both older rodents and adults (DeSouza *et al.,* 2000; Durrant *et al.,* 2009; Fleenor *et al.,* 2010; Jablonski *et al.,* 2015; Lesniewski *et al.,* 2011; Trott *et al.,* 2009). We found that VR attenuated T cell and macrophage infiltration in both the aorta and mesenteric vasculature. In accord with our previous observations (Lesniewski *et al.,* 2011) we found that aortic macrophage infiltration was attenuated with VR; however, in contrast, in the present study we found that VR also attenuated aortic T cell infiltation. This may be due to the differences between quantifying immune cell infiltration by histology of arterial sections vs. flow cytometry of the whole artery. In contrast to CR, VR did not attenuate age-related accumulation of B cells in the aorta and mesentery. Because both exercise and CR improve arterial function with age, T cells and macrophages might play a relatively greater role in age-related arterial inflammation and dysfunction than B cells. In addition, it is possible that lifelong CR prevents arterial accumulation of B cells whereas 8 weeks of VR is not sufficient to reverse already existing B cell infiltration. Notably, exercise slows T cell aging in rodents and aerobic fitness is inversely related to circulating end differentiated T effector memory cells in older adults (Spielmann *et al.,* 2011; Woods *et al.,* 2003). This study demonstrates that in addition to the well described mechanisms of vasoprotection by CR and VR, these interventions also reduce vascular immune cell infiltration. Interestingly, both CR and VR reduced infiltration of both T cell subsets in the aorta but only $CD8^+$ T

cells in the mesentery. The mechanism behind these differences and the respective roles of these T cell subtypes in the aged vasculature are unknown. Further, the mechanisms by which CR and exercise might modulate the interaction between the immune system and vasculature are important topics for future study.

The mice in the present study ran considerably less than old B6D2F1 mice in previous reports (Durrant *et al.,* 2009; Lesniewski *et al.,* 2011). Although voluntary wheel running does not allow us to control exercise duration or intensity we chose this modality as forced treadmill training in rodents has been shown to activate neuronal stress pathways that can also mediate arterial immune cell infiltration and activation (Marvar *et al.,* 2010; Marvar *et al.,* 2012; Yanagita *et al.,* 2007). Although we do not know the relative intensity of exercise in the present study, this data suggests physical activity beyond that of cage control mice is sufficient to reduce arterial immune cell infiltration. Whether greater amounts of physical activity might further decrease age-related arterial immune cell infiltration is an interesting question. A limitation of the present study is that due to attrition typical of older mice (Forster *et al.,* 2003) we did not achieve our target sample size of 8 per group in the OVR group as calculated based on data from Lesniewski et al., 2011. Despite this limitation, the major conclusions of this investigation remain. We found significant differences in total aortic immune cells, T cells and macrophages when the OVR group was compared to ONC. We also found differences in mesenteric T cells and macrophages when comparing these same groups.

4.4 Aging and immune cell gene expression

We hypothesized that aging would increase proinflammatory immune cell gene expression and that this would be ameliorated by CR or VR. We found that $CD4^+$ T cells exhibited greater Tbet and FoxP3 expression with age and that this was not altered by CR or VR. Tbet is the primary transcription factor that leads naïve T cells down the proinflammatory Th1 linage and to produce IFN-γ. In stimulated, *in vitro* conditions CD4+ cells from old mice produce more IFN-γ compared to cells from young mice (Hobbs *et al.,* 1993). Although we found no change in splenic T cell IFN-γ gene expression, it is possible that these cells are incompletely or undifferentiated in the spleen and become fully differentiated as they migrate to peripheral tissues where they induce inflammation and dysfunction. We also found that aging resulted in greater expression of the transcription factor FoxP3, which drives cells to a T regulatory phenotype. These cells orchestrate the resolution of inflammation and are typically considered anti-inflammatory. However, T regulatory cells have recently been shown to mediate age-related insulin resistance (Bapat *et al.,* 2015), suggesting that aging may result in a shift to a pathogenic phenotype in these cells.

Contrary to our hypothesis, we found a no changes in $CD8^+$ T cell gene expression for proinflammatory or cytotoxic genes. In B cells, we found a decrease in gene expression for the proinflammatory cytokine IFN-γ, no change for TNF-α and a trend for increased expression of the anti-inflammatory cytokine, IL-10. In mesenteric macrophages, TNF-α expression was decreased with age. In contrast to our findings, splenic B cells and visceral adipose resident macrophages from aged mice have been shown to secrete more TNF-α *in vitro* (Lumeng *et al.,* 2011; Ratliff *et al.,* 2013). This difference may be due differences in

methodology, e.g. qPCR vs. cytokine secretion and/or unstimulated primary cells vs. stimulated cells in culture. It should be noted that despite exhibiting decreased or unchanged gene expression for proinflammatory cytokines, the magnitude of change in arterial infiltrating immune cells with age (~5-fold increase in both aorta and mesentery) could lead to increased local proinflammatory cytokine concentrations contributing to arterial dysfunction despite lower levels of cytokine gene expression from individual cells.

4.5 Conclusions

In summary, our data indicate that aging results in a profound infiltration of T cells, B cells and macrophages in both the aorta and mesenteric vasculature. Lifelong CR almost completely normalized age-related arterial immune cell infiltration and VR attenuated T cell and macrophage but not B cell infiltration. These are the first data to demonstrate that immune cells *per se* contribute to age-related arterial inflammation in both large elastic arteries and the resistance vasculature. We are also the first to report that CR can prevent and VR partially reverse age-related arterial immune cell infiltration. Important topics for future study include; whether and which subtype(s) of arterial infiltrating immune cells directly contribute to age-related arterial dysfunction, mechanisms by which these cells are recruited to the artery and, the mechanisms by which CR and VR attenuate arterial immune cell infiltration.

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Abbreviations

YNC	young normal chow
ONC	old normal chow
OCR	old caloric restriction
OVR	old voluntary running
CD	cluster of differentiation
CVD	cardiovascular disease
EDD	endothelium dependent dilation
VAMC-SLC	Veteran’s Affairs Medical Center-Salt Lake City
TNF-α	Tumor necrosis factor-α

IFN-γ	
IL	interleukin
Th1	T helper 1

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Highlights

1. Aging is associated with increases in both large- and resistance-artery infiltrating leukocytes
2. Age-related arterial leukocyte infiltration is normalized by caloric restriction
3. Age-related arterial leukocyte infiltration is attenuated by voluntary running
4. Aging results in alterations in $CD4^+$ T cell transcription factor expression
5. Aging results in decreased proinflammatory cytokine gene expression in B cells and macrophages

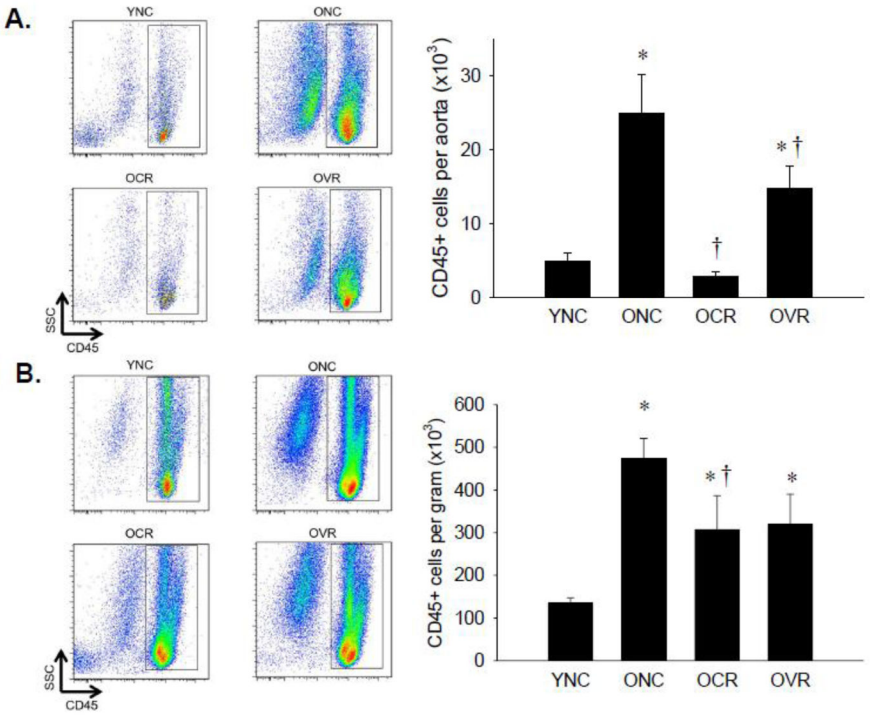

Figure 1. Leukocyte infiltration of aorta and mesenteric vascular arcade

Aortas (A) and mesenteric vascular arcade (B) from young normal chow (YNC), old- (O) normal chow (NC), voluntary running (VR) and calorie restricted (CR) mice were digested to a single cell suspension and stained with antibodies against CD45 to assess total leukocytes. Representative flow cytometry plots are shown on the left of each panel, summary data is shown on the right. n = 5–11/group. Differences were assessed with one-way ANOVA with LSD post hoc tests. * different from YNC, † different from ONC, $p \leq 0.05$. Data are means ± SEM.

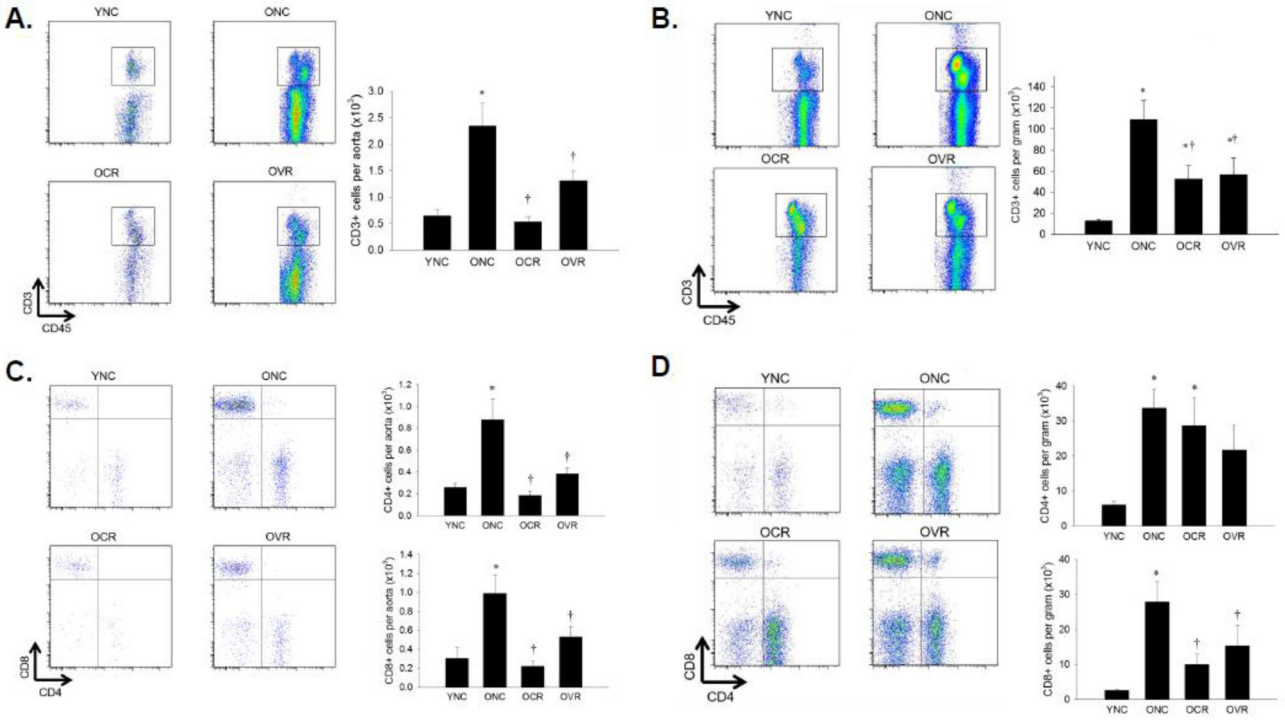

Figure 2. T cell infiltration of aorta and mesenteric vascular arcade

Aortas (A) and mesenteric vascular arcade (B) from young normal chow (YNC), old- (O) normal chow (NC), voluntary running (VR) and calorie restricted (CR) mice were digested to a single cell suspension and incubated with antibodies against CD45 and CD3 to assess total leukocytes $CD4^+$ and $CD8^+$ T cell populations were assessed in the same groups by addition of antibodies against CD4 and CD8 in aorta (C) and mesentery (D). Representative flow cytometry plots are shown on the left of each panel, summary data is shown on the right. n = 5–11/group. Differences were assessed with one-way ANOVA with LSD post hoc tests. * different from YNC, † different from ONC, $p \leq 0.05$. Data are means ± SEM.

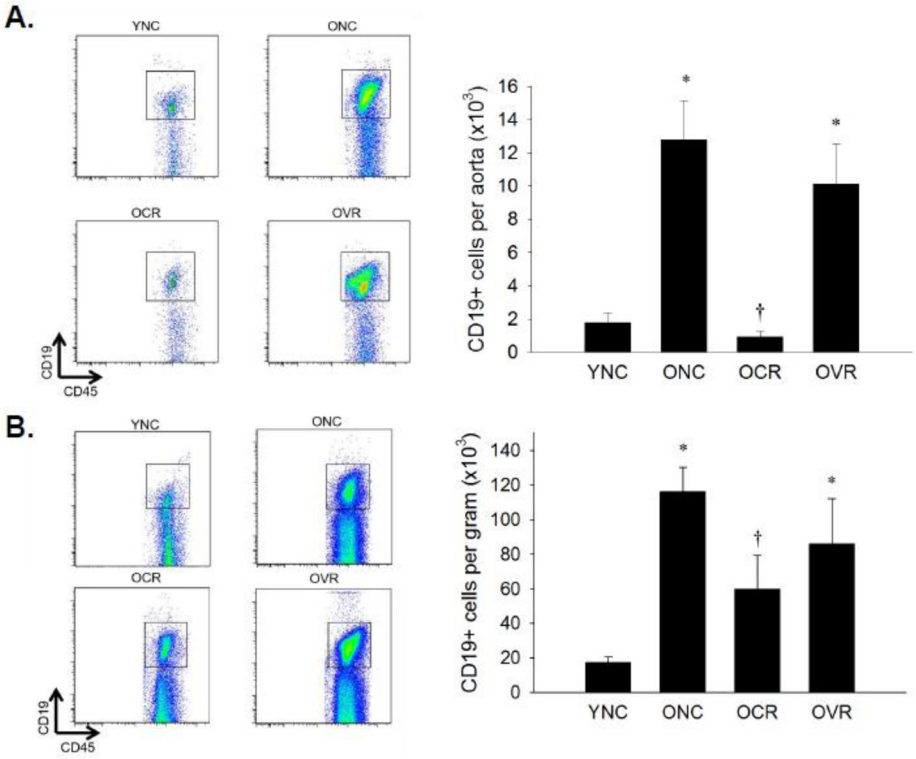

Figure 3. B cell infiltration of aorta and mesenteric vascular arcade

Aortas (A) and mesenteric vascular arcade (B) from young normal chow (YNC), old- (O) normal chow (NC), voluntary running (VR) and calorie restricted (CR) mice were digested to a single cell suspension and stained with antibodies against CD45 and CD19 to assess B cell infiltration. Representative flow cytometry plots are shown on the left of each panel, summary data is shown on the right. n = 5–11/group. Differences were assessed with one-way ANOVA with LSD post hoc tests. * different from YNC, † different from ONC, $p \leq 0.05$. Data are means ± SEM.

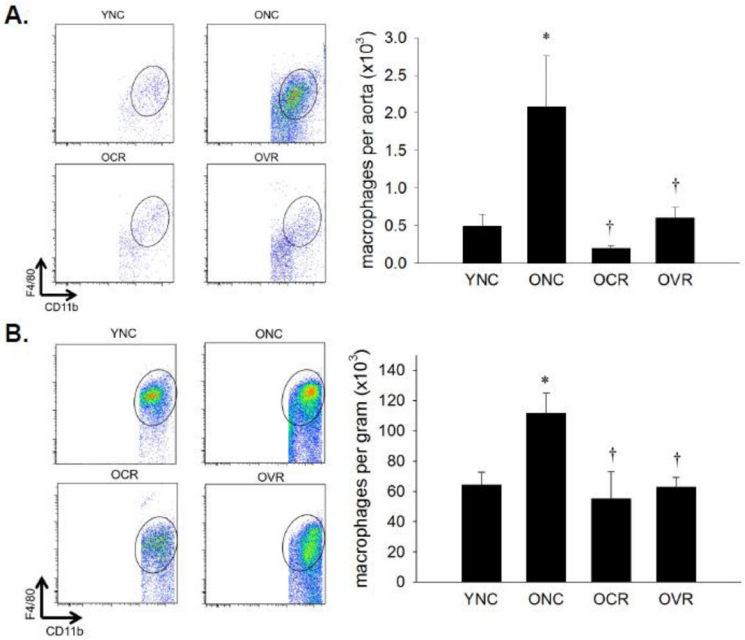

Figure 4. Macrophage infiltration of aorta and mesenteric vascular arcade

Aortas (A) and mesenteric vascular arcade (B) from young normal chow (YNC), old- (O) normal chow (NC), voluntary running (VR) and calorie restricted (CR) mice were digested to a single cell suspension and stained with antibodies against CD45, CD11b and F4/80 to assess macrophage infiltration. Representative flow cytometry plots are shown on the left of each panel, summary data is shown on the right., n = 5–11/group. Differences were assessed with one-way ANOVA with LSD post hoc tests. * different from YNC, † different from ONC, $p \leq 0.05$. Data are means ± SEM.

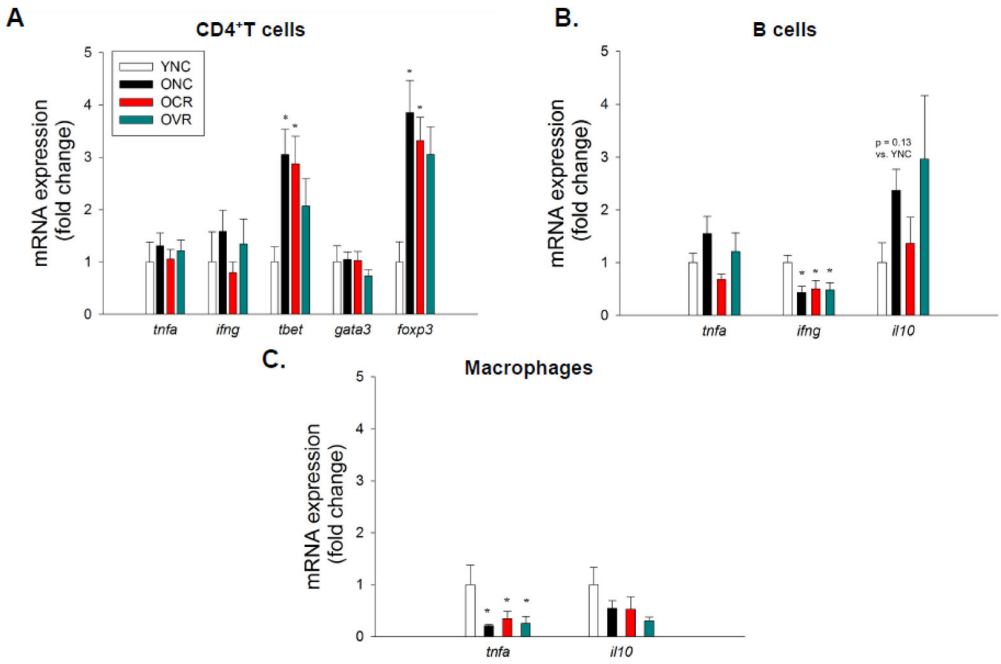

Figure 5. Immune cell pro- and anti-inflammatory gene expression. Splenic

$CD4^+$ T cells (A) and B cells (B) and mesenteric macrophages (C) were isolated using Flow Activated Cell Sorting (FACS). Following RNA isolation and cDNA synthesis qPCR was employed to assess gene expression for inflammatory cytokines TNF-α (*tnfa*), IFN-γ (*ifng*), anti-inflammatory cytokine Interleukin-10 (*il10*) and T cell transcription factors Tbet (*tbet*), GATA3 (*gata3*) and FoxP3 (*foxp3*). 18s RNA was used as a reference gene and mRNA fold change was calculated using the ΔΔCt method. n = 4–11/group. Differences were assessed with one-way ANOVA with LSD post hoc tests. * different from YNC, † different from ONC, p 0.05. Data are means ± SEM.

Table 1

Animal body and tissue mass

Group	YNC	ONC	OCR	OVR
Body (g)	33.7 ± 0.7	36.4 ± 1.0 *	29.2 ± 0.8 †	35.5 ± 1.4
Mesentery (g)	0.22 ± 0.01	0.16 ± 0.01 *	0.11 ± 0.01 *	0.24 ± 0.04 †
Heart (g)	0.18 ± 0.009	0.22 ± 0.007 *	0.16 ± 0.004 †	0.20 ± .008 *†
Soleus (g)	0.012 ± 0.0009	0.011 ± 0.0005	0.009 ± 0.0004 *	0.014 ± 0.0010 †
Visceral adipose (g)	0.62 ± 0.05	0.33 ± 0.03 *	0.24 ± 0.03 *	0.42 ± 0.09 *

YNC: young normal chow, ONC: old normal chow, OCR: old caloric restricted, OVR: old voluntary running. n = 5–11/group. Differences were assessed with one way ANOVA and LSD post hoc tests.

*Different from YNC,

†different from ONC, $p < 0.05$

Data are means ± SEM

Table 2

Splenic leukocyte characteristics

Group	YNC	ONC	OCR	OVR
Spleen mass (g)	0.097 ± 0.001	0.124 ± 0.042	0.045 ± 0.002†	0.112 ± 0.024
Total number of CD45^{+} cells (x10^{7})	4.1 ± 0.6	7.4 ± 1.2	4.2 ± 0.7	4.9 ± 0.5
% CD3^{+} cells	18.5 ± 1.0	14.1 ± 1.7	16.2 ± 2.5	17.1 ± 2.5
CD4/CD8 ratio	1.23 ± 0.06	1.00 ± 0.13	1.05 ± 0.29	1.04 ± 0.26
% CD19^{+} cells	60.8 ± 0.8	63.6 ± 5.4	61.9 ± 2.8	67.2 ± 3.5
% CD11b^{+} cells	7.3 ± 1.2	10.3 ± 1.2	13.4 ± 1.0*	9.5 ± 1.3

YNC: young normal chow, ONC: old normal chow, OCR: old caloric restricted, OVR: old voluntary running. n = 5–11/group. Differences were assessed with one way ANOVA and LSD post hoc tests.

* Different from YNC, $p < 0.05$

Data are means ± SEM.