Published in final edited form as:

Cytokine. 2017 August ; 96: 102–106. doi:10.1016/j.cyto.2017.03.008.

Selected Life-extending Interventions Reduce Arterial CXCL10 and Macrophage Colony-stimulating Factor in Aged Mouse Arteries

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Abstract

Cardiovascular disease (CVD) is the leading cause of death in the industrialized world. Aging is the most predictive risk factor for CVD and is associated with arterial inflammation which contributes to increased CVD risk. Although age-related arterial inflammation has been described in both humans and animals, only a limited number of inflammatory mediators, cytokines and chemokines have been identified. In this investigation we sought to determine whether lifespan extending interventions, including crowded litter early life nutrient deprivation (CL), traditional lifelong caloric restriction (CR) and lifelong Rapamycin treatment (Rap) would attenuate age-related arterial inflammation using multi analyte profiling. Aortas from Young (4–6 months), Old (22 months), Old CL, Old CR and Old Rap mice were homogenized and cytokine concentrations were assessed using Luminex Multi Analyte Profiling. Chemokines involved in immune cell recruitment, such as CCL2, CXCL9, CXCL10, GMCSF and MCSF, were increased in Old vs. Young ($p < 0.05$). The age-related increase of CXCL10 was prevented by CR ($p < 0.05$ vs. Old). MSCF concentrations were lower in aortas of Rap treated mice ($p < 0.05$ vs. Old). Interleukins (IL), IL-1α, IL-1β and IL-10, were also greater in Old vs. Young mice ($p < 0.05$). These data demonstrate selected lifespan extending interventions can prevent or limit age-related increases in selected aortic chemokines.

Keywords

cytokine; chemokine; caloric restriction; rapamycin; aorta; interleukin

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1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the industrialized world. Aging is the most predictive risk factor for CVD and most CVD are a result of arterial dysfunction [1]. In particular, arterial aging manifests itself though decreased endothelium dependent dilation (EDD) and increased stiffness of the large arteries [1–5]. Arterial inflammation is also observed with age in both humans and animals and is associated with functional impairment [3, 6–11]. Age-related arterial inflammation appears to involve endothelial cells [7], smooth muscle cells [8] and accumulation of immune cells in the perivascular space [10]. Treatment with anti-inflammatory interventions has been shown to improve arterial function in older adults [12, 13]. Despite these observations, there is limited information about how arterial chemokines and cytokines may be altered by age.

Crowded litter early life nutrient deprivation (CL) [14], lifelong caloric restriction (CR) [15, 16], and lifelong rapamycin treatment (Rap) [17–19] have all been shown to extend lifespan in mice. Notably, CR and Rap have also been shown to improve age-related vascular dysfunction including improvements in large artery stiffness and EDD [20–23]. In this investigation, we sought to gain a more comprehensive profile of the cytokines and chemokines produced by the aged artery through the use of Multi-Analyte Profiling (MAP). In addition, we sought to determine whether lifespan extending interventions CL, CR or Rap would prevent age-related changes in the arterial inflammatory profile.

We hypothesized that aging would result in increased arterial concentrations of pro-inflammatory cytokines/chemokines and decreased concentrations of anti-inflammatory cytokines. Further, we hypothesized that life extending interventions would prevent or retard these age-related changes in the arterial inflammatory profile.

2. METHODS

2.1 Mice

All procedures conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Committee on Use and Care of Animals at the University of Michigan, University of Utah, and Veteran's Affairs Medical Center-Salt Lake City (VAMC-SLC). All mice were housed in standard mouse cages with *ad libitum* access to water and food (with exceptions noted below) on a 12:12 light:dark cycle. Young mice were housed at the VAMC-SLC animal facilities and all old mice regardless of treatment group were housed at the University of Michigan animal facilities. Five groups of mice were employed in this study; 1) Young (4–6 month) B6D2F1 mice, n = 8. 2) Old (22 month) genetically heterogeneous offspring of CB6F1 female and C3D2F1 male (UM-HET3) mice, n = 29. 3) Old Crowded litter (CL) UM-HET3 mice, n = 10. Litters for these mice were culled to eight, and an additional four mice from separate litters were added, resulting in a 50% increase in litter size as previously described [14, 24]. 4) Old Caloric Restriction (CR) mice, n = 19. Starting at 4–5 weeks of age these mice received 66–70% of the food a mouse of similar age and sex would consume *ad lib* as described [25]. 5) Old Rapamycin (Rap) treated mice, n = 23. Starting at 9 weeks of age mice were provided with a diet containing 14 ppm (mg of drug per kg of food). The old control group is the largest (n = 29) as we pooled control mice for

each anti-aging intervention. Dates of birth for all old mice were between October 19, 2009 and March 18, 2010, old mice were sacrificed at 22 months of age. Young mice had dates of birth between January 4, 2011 and April 25, 2014 and were sacrificed at 4–6 months of age.

At sacrifice aortas were dissected, homogenized and lysed for protein analysis as previously described [26, 27]. Aortic protein concentrations were determined by BCA protein Assay (ThermoFisher). A total of 7μg of aortic protein was loaded onto two separate Milliplex MAP mouse cytokine/chemokine plates (Millipore), the plates was prepared according to manufacturer's protocols and read on a Luminex MAGPIX system. From each sample, the following cytokine concentrations were assessed: CC ligand (CCL)2, CCL3, CCL4, CCL5, CCL11, CXC ligand (CXCL)2, CXCL5, CXCL9, CXCL10, Granulocyte Colony Stimulating Factor (GCSF), Granulocyte Macrophage Colony Stimulating Factor (GMCSF), Interferon (IFN)-γ, Interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-3, IL-5 IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, Leukemia Inhibitory Factor (LIF), Macrophage Colony Stimulating Factor (MCSF), Tumor Necrosis Factor (TNF)-α and Vascular Endothelial Growth Factor (VEGF).

2.2 Statistical Analysis

As individual data points for a number of analytes were at or below the level of detection of the MAP assay and because the data for several analytes were not normally distributed, differences were assessed with the Kruskal-Wallis one-way ANOVA on ranks. Using this analysis allowed for appropriate statistical comparison where calculation of mean and standard error was not possible. Dunn's pairwise comparison *post hoc* test was employed to determine group differences. Adjusted p values of $p < 0.05$ were considered statistically significant.

3. RESULTS

The following cytokines were undetectable in the majority of aortas regardless of group: CCL4, CXCL5, GCSF, IL-3, IL-4, IL-5, IL-7 IL-12p40, IL-15, IL-17, LIF and VEGF (data not shown). CCL3, CCL5, CCL11, CXCL2, IFN-γ, IL-2, IL-6, IL-8, IL-9 and IL-12p70 did not exhibit significant increases in Old compared to Young control aortas (Table 1). CCL11 was increased in Old CL vs. Young ($p < 0.05$, Table 1). CCL3 and IL-8 were both increased in Old CR compared to Young ($p < 0.05$, Table 1). CCL5 and IL-9 were both increased in Old CL compared to Old CR.

Chemokines principally involved in immune cell recruitment and activation CCL2, CXCL9, CXCL10, GMCSF and MCSF were increased in Old aortas vs. Young (Figure 1). CCL2 and GMCSF were not altered by any intervention (Figure 1A, D). The age-related increase in CXCL10 was abolished by CR ($p < 0.05$ vs. Old, Figure 1C). MCSF was not altered by CL or CR but was similar to Young in the Old Rap group ($p < 0.05$ vs. Old, Figure 1E). Aortic concentrations of IL-1α, IL-1β and IL-10 were increased with age (Young vs. Old $p < 0.05$, Figure 2), these cytokines were not altered by any of the life extending interventions. To assess whether these observations were confounded by differences in the two multiplex plate runs, each plate was analyzed separately. We found that directional changes were preserved

regardless of which plate was excluded, to reach statistical significance some differences required inclusion of both plates.

4. DISCUSSION

The major findings of this study are: 1) CCL2, CXCL9, CXCL10, GMSCF and MCSF, chemokines principally involved in immune cell recruitment and activation are increased with age in the mouse aorta. 2) Age-related increases in CXCL10 and MCSF were abolished by CR and Rap, respectively. 3) Interleukins IL-1α, IL-1β and IL-10 were increased with age, but were not impacted by lifespan extending interventions. These results demonstrate that aging is associated with increased aortic concentrations of multiple chemokines involved in immune cell recruitment and that in some cases, this increase is prevented by selected life extending interventions. Further, aging is associated with increases in both pro- (IL-1α and β) and anti-inflammatory (IL-10) Interleukins.

In the present investigation we found that aging is associated with increased aortic concentrations of CCL2, CXCL9, CXCL10, GMCSF and MSCF. CCL2, also known as Monocyte Chemoattractant Protein (MCP)-1 serves to recruit monocytes and T cells to sites of inflammation [28]. CXCL9 and CXCL10 also known as Monokine Induced by γ-Interferon (MIG) and Interferon-γ-induced Protein 10 (IP-10), respectively are closely related T cell chemoattractants [29]. GMCSF and MCSF are monocyte growth factors that induce differentiation, proliferation and recruitment of both granulocytes and macrophages and macrophages only, respectively [30].

Our observation that CCL2 is increased in the aged mouse aorta is consistent with previous observations showing increased CCL2 in aged human endothelial cells [7] and whole arteries [9] as well as rat aorta [11] and primary vascular smooth muscle cells [8]. Further, higher plasma levels of CCL2 along with CXCL9 and CXCL10 have been observed in older adults compared to young controls [31, 32]. Plasma GMSCF is not altered in older adults compared to young controls [33]; however, endothelial cells produce greater levels of GMSCF under conditions of replicative senescence [34]. Relatively little is known about MCSF and aging. This study is, to our knowledge, the first to show increased production of CXCL9, CXCL10, GMCSF and MCSF in the aged artery. The production of the above cytokines by the artery may contribute to increased arterial immune cell infiltration that has been observed with age [10,35]. Whether these immune cells directly mediate age-related arterial dysfunction is unknown, but both T cells and macrophages are implicated in mediating arterial dysfunction in experimental hypertension [36, 37]. Together these observations suggest that increased production of chemokines that recruit immune cells is an important component of arterial inflammation with age.

We have previously found that lifelong CR prevents age-related arterial dysfunction [20, 22] and accumulation of T cells in both aorta and the mesenteric vasculature [35]. In the present investigation, we found that age-related increases CXCL10, which recruits T cells, was prevented by CR. We also found that treatment with Rap lowered MCSF, which recruits and serves as a growth factor for macrophages. Consistent with this observation, aortic perivascular macrophages increase with age [10, 35], and Rap treatment improves arterial

function in old mice [21]. The finding that some of the lifespan extending interventions can prevent age-related increases in aortic chemokines that mediate differentiation and recruitment of inflammatory cells represents a potential mechanism by which these interventions might preserve arterial health.

We also found that concentrations of aortic IL-1α, IL-1β and IL-10 were increased with age. IL-1α and IL-1β are potent proinflammatory cytokines and modulators of innate immunity. These cytokines are produced by numerous cell types and increased circulation of IL-1α and IL-1β lead to fever, leukocyte migration and activation and, increased expression of endothelial adhesion molecules [37]. IL-1α has been shown to induce production of senescence associated secretory phenotype (SASP) cytokines (including CCL2) in cultured vascular smooth muscle cells in autocrine manner [38] and thus, may contribute to vascular inflammation with age. The increase in IL-1β is in accord with a previous observation by our group showing increased aortic IL-1β concentrations in with age in mice [10]. IL-10 is an anti-inflammatory cytokine and mice deficient in IL-10 develop arterial dysfunction at a younger age than wild type controls [39]. This suggests that the age-related increase in IL-10 may be a compensatory response in attempt to preserve arterial function with age.

Interestingly, changes in IFN-γ, IL-6, IL-8 and TNF-α described in previous investigations [6, 8–10] were absent in the present study. This may be partly explained by differences in methodology (i.e. measurement of gene expression rather than cytokine concentration, using stimulation *in vitro* to evoke cytokine release etc.). Further, in the present study old mice were sacrificed at 22 months, 4–5 months before median survival age [19] in the UM-HET3 mice. In a report from our laboratory, increases in IFN-γ, IL-6 and TNF-α were observed at 31 months in B6D2F1 mice which corresponds to median survival age [10]. Thus, it is conceivable that the changes observed in the present study occur with age but precede increases in proinflammatory cytokines and chemokines observed in very old mice. A limitation of the present study is that the young mice were B6D2F1 mice housed at the VAMC-SLC whereas the old mice were the UM-HET3 (offspring of CB6F1 female and C3D2F1 male) housed at the University of Michigan. Thus, it is possible that the aging differences are confounded by differences in strain and housing. In accord with the present study, we have previously found that arterial IL-1β is increased with age in B6D2F1 mice [10]. The literature also indicates that arterial CCL2 is increased with age in both rodents and humans [7–9, 11]. It should be noted that CR decreases CXCL10 and Rap treatment decreases GMCSF compared to strain matched old controls.

In the present investigation, we found that aging results in increased aortic concentrations of CCL2, CXCL9, CXCL10, GMSCF and MCSF, all cytokines involved in recruitment of immune cells. We found that CR and Rap modulated age-related changes in aortic CXCL10 and MCSF, respectively. Lastly, we found that both pro-(IL-1α and IL-1β) and anti-inflammatory (IL-10) interleukins were increased with age and not affected by life extending interventions. These results suggest that upregulation of cytokines that recruit immune cells is an important aspect of age-related arterial inflammation. Whether these cytokines directly contribute to increased arterial immune cell infiltration and how this might affect arterial function with age are unknown and important topics for future study. The interplay and precise roles of these cytokines are also important topics for further study.

Acknowledgments

The authors would like to thank Richard A. Miller (University of Michigan) for providing the tissues from aged mice and mice exposed to interventions that extend lifespan. This work was supported by National Institute of Aging award numbers K02AG045339, R01AG050238, R01AG040297, R01AG048366, R01-022303, the Glenn Foundation for Medical Research, and by Merit Review Award 1I01BX002151 from the United States (U.S.) Department of Veterans Affairs Biomedical Laboratory Research and Development Service. The contents do not represent the views of the U.S. Department of Veterans Affairs or the United States Government.

Glossary

CVD	Cardiovascular disease
EDD	endothelium dependent dilation
CL	crowded litter
CR	caloric restriction
Rap	Rapamycin
IL	interleukin
VAMC-SLC	Veteran's Affairs Medical Center-Salt Lake City
CCL	CC ligand
CXC	CXC-ligand
GCSF	Granulocyte Colony Stimulating Factor
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
LIF	Leukemia Inhibitory Factor
MCSF	Macrophage Colony Stimulating Factor
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

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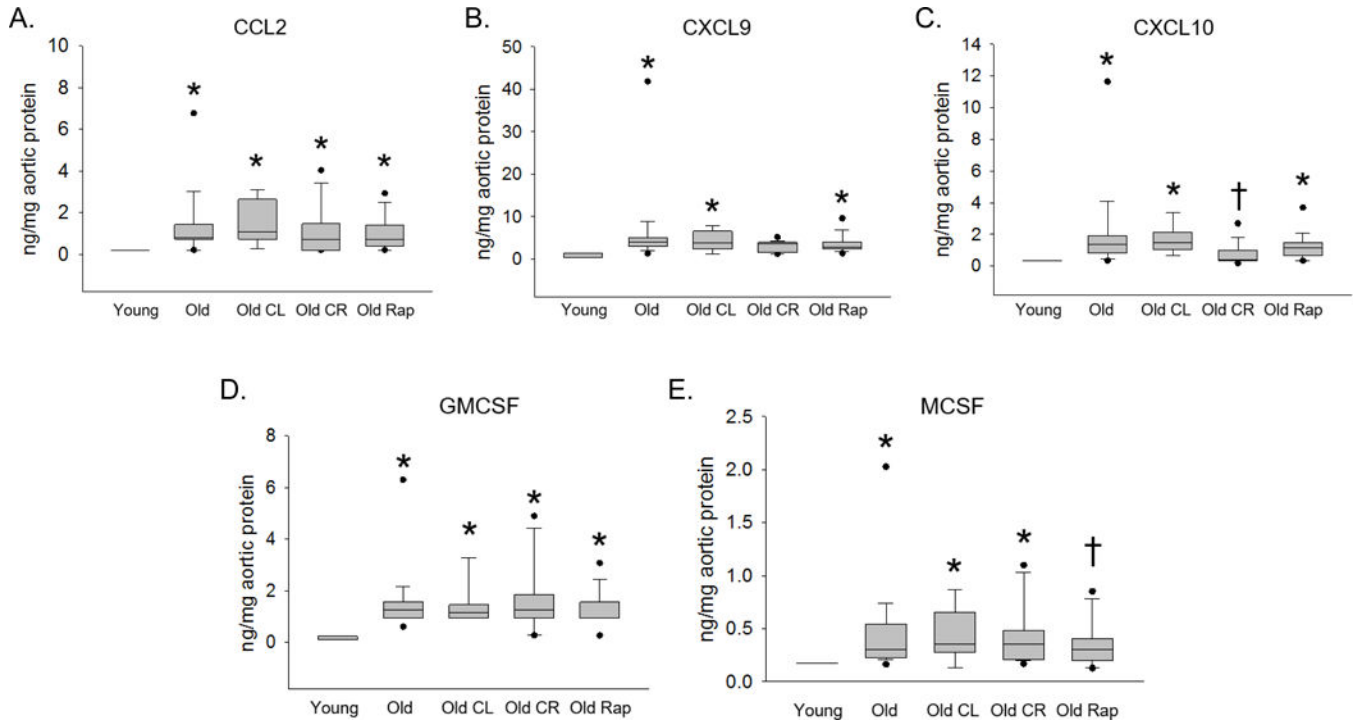

Figure 1. Age-related increases in aortic concentrations of cytokines involved in immune cell recruitment

Aortas from Young (4–6 months), Old (22 months), Old crowded litter (CL), Old caloric restriction (CR) and Old Rapamycin treated (Rap) mice were homogenized and chemokine and cytokine concentrations were assessed using Luminex Multi Analyte Profiling data for chemokines CCL2 (A), CXCL9 (B), CXCL10 (C), GMCSF (D) and MCSF (E) are shown. n = 8–29/group. Differences were assessed with Kruskal-Wallis one-way ANOVA on ranks and Dunn's pairwise comparison *post hoc* test. An asterisk (*) indicates different from Young, † different from Old, $p \leq 0.05$. Data are expressed as box and whisker plots where the horizontal line within the box indicates the median, boundaries of the box indicate the 25^{th}- and 75^{th} -percentile, the whiskers indicate the 5^{th}- and 95^{th}-percentile, dots represent outlying values.

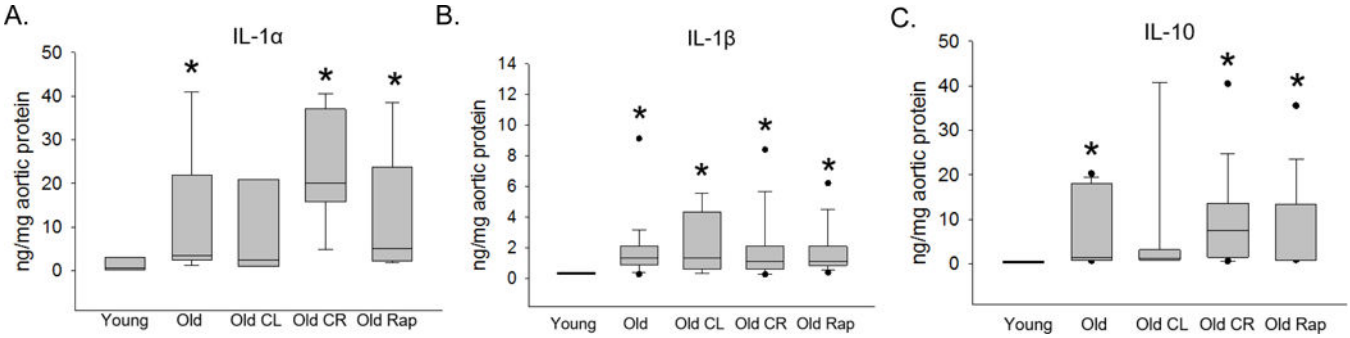

Figure 2. Age-related increases in aortic concentrations of selected interleukins family

Aortas from Young (4–6 months), Old (22 months), Old crowded litter (CL), Old caloric restriction (CR) and Old Rapamycin treated (Rap) mice were homogenized and chemokine and cytokine concentrations were assessed using Luminex Multi Analyte Profiling data for Interleukin (IL)-1α (A), IL-1β (B) and IL-10 (C) are shown. n = 8–29/group. Differences were assessed with Kruskal-Wallis one-way ANOVA on ranks and Dunn's pairwise comparison *post hoc* test. An asterisk (*) indicates different from Young, † different from Old, $p \leq 0.05$. Data are expressed as box and whisker plots where the horizontal line within the box indicates the median, boundaries of the box indicate the 25^{th}- and 75^{th} -percentile, the whiskers indicate the 5^{th}- and 95^{th}-percentile, dots represent outlying values.

Table 1

Aortic Chemokine/Cytokine Concentrations (ng/mg aortic protein)

	Young	Old	Old CL	Old CR	Old Rap
n	8	29	10	19	23
CCL3	1.00 (1.00, 1.51)	3.40 (0.83, 14.67)	1.45 (0.66, 6.73)	10.34 (6.99, 13.30) *	3.04 (0.09, 8.73)
CCL5	0.33 (0.33, 0.34)	0.33 (0.33, 2.59)	0.33 (0.33, 0.62)	1.36 (0.47, 2.49) †	0.33 (0.33, 1.99)
CCL11	0.30, (0.30, 6.61)	9.32 (3.73, 13.82)	13.44 (7.17, 16.29) *	4.41 (3.26, 5.61)	10.91 (3.77, 13.09)
CXCL2	0.64 (0.64, 1.29)	1.50 (1.06, 2.33)	1.56 (1.06, 2.99)	1.50 (1.06, 3.14)	1.06 (1.06, 2.33)
IFN-γ	0.49 (0.49, 0.49)	0.49 (0.34, 0.49)	0.35 (0.34, 0.49)	0.49 (0.49, 0.49)	0.49 (0.34, 0.49)
IL-2	0.27 (0.27, 0.27)	0.30 (0.21, 0.69	0.27 (0.07, 0.62)	0.36 (0.27, 0.63)	0.44 (0.27, 0.61)
IL-6	0.39 (0.39, 0.69)	0.48 (0.33, 1.03)	0.33 (0.33, 0.50)	0.66 (0.39, 0.76)	0.39 (0.33, 0.54)
IL-8	0.86 (0.41, 1.13)	2.00 (0.63, 3.27)	0.89 (0.61, 1.63)	2.19 (1.60, 2.96) *	1.20 (0.70, 2.10)
IL-9	46.92 (31.64, 64.79)	30.0 (17.64, 121.49)	20.57 (10.76, 65.81)	97.00 (56.11, 131.83) †	29.29 (17.00, 94.41)
IL-12p70	0.24 (0.21, 0.36)	0.74 (0.23, 1.07)	0.51 (0.31, 0.87)	0.74 (0.40, 0.94) *	0.50 (0.22, 0.74)

CL: crowded litter, CR: caloric restriction, Rap: Rapamycin. Differences were assessed with Kruskal-Wallis one-way ANOVA with Dunn's pairwise comparisons.

*Different from Young,

†different from Old CL, $p < 0.05$ Data are medians (25th percentile, 75th percentile).