ABSTRACT

Title of Document: EFFECTS OF CARDIOVASCULAR DISEASE AND PHYSICAL INACTIVITY ON THE PARACRINE FUNCTION OF CIRCULATING ANGIOGENIC CELLS.

Rian Q. Landers-Ramos, Doctor of Philosophy, 2015

Directed by: Professor James M. Hagberg, Ph.D.
Department of Kinesiology

Cardiovascular disease (CVD) is the leading cause of death in developed countries. Traditional cardiovascular risk factors account for only a fraction of events related to CVDs, emphasizing the need for investigations into more novel risk factors. Circulating angiogenic cells (CACs) are involved in the repair and maintenance of the vascular endothelium and function mainly through paracrine mechanisms. The studies presented in this dissertation provide new insight into differences in the paracrine actions of CACs as a function of habitual physical activity and CVD. The first study presented identifies, for the first time, that secreted factors from CD34+ and CD34-/CD31+ CACs affect HUVEC tube formation as a function of habitual physical activity. Study #1 identifies inflammatory proteins S100A8 and S100A9 as major factors contributing to the depressed tube formation observed when using CD34-/CD31+ conditioned media (CM) from inactive younger adults compared to endurance-trained athletes. The second study aimed to confirm the effects of S100A8 and S100A9 in CD34-/CD31+ CM on HUVEC tube formation in CVD patient populations compared to endurance-trained athletes. Study #2 found that the CM from non-ST- segment elevation myocardial infarction (NSTEMI) patient CD34-/CD31+ CACs impaired tube formation compared to athletes' CM, and that pretreatment of HUVECs with an inhibitor for TLR4, a major receptor for S100A8 and S100A9, rescued tube formation to the levels observed when using CD34-/CD31+ CM from athletes. Higher S100A8 and S100A9 content was found in the CM of NSTEMIs compared to athletes. Finally, the study #2 mechanistically demonstrated the direct role of S100A8 and S100A9 on tube formation using recombinant S100A8 and S100A9 and confirmed that these actions were mediated by TLR4. Preliminary data in study #2 suggest that cell surface markers on selected CD34-/CD31+ CACs are inherently different between NSTEMI patients and endurance-trained athletes with lower presence of T-cell and monocyte markers on the CD34-/CD31+ CACs of NSTEMI patients. Collectively, the two studies presented in this dissertation demonstrate that both physical inactivity and CVD alter the paracrine actions of CD34-/CD31+ CACs which in turn impair HUVEC tube formation. These findings are of particular importance as new methods to improve CAC function for therapeutic purposes are being developed.

EFFECTS OF CARDIOVASCULAR DISEASE AND PHYSICAL INACTIVITY ON THE PARACRINE FUNCTION OF CIRCULATING ANGIOGENIC CELLS

By

Rian Q. Landers-Ramos

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2015

Advisory Committee:

Professor James M. Hagberg, Ph.D., Chair

Assistant Professor Eva R. Chin, Ph.D.

Assistant Professor Steven J. Prior, Ph.D.

Associate Professor Espen E. Spangenburg, Ph.D.

Professor David Mosser, Ph.D., Dean's Representative

© Copyright by

Rian Quinn Landers-Ramos

2015

Dedication

I dedicate this dissertation to my father, Michael M. Landers, who encouraged me to pursue my Ph.D. and always emphasized the importance of doing work that makes you happy.

Acknowledgements

There are so many people who have influenced me both personally and career-wise who deserve thanks as I reach this major milestone. I especially want to acknowledge my parents for their love and support through out my life and this academic journey. Thank you for emphasizing the importance of education, hard work, and most importantly finding work that I love. Thank you to my sisters, Caolan, Devon, Taryn, and Shannon. The dedication that you all have toward your families, careers, and to live healthy lives help to motivate me every day. Thank you to my husband, Mike, for your ongoing support, love, and patience and for bringing me dinner and the occasional visit with a puppy on nights I had to work late in the lab. I love you all so much!

I want to express a huge thank you to my mentor, friend, and occasional psychotherapist, Dr. Jim Hagberg. You have taught me more than I could have ever imagined over the last 5 years. Your dedication to your work and to teaching and mentoring students is an inspiration. You have taught me important lessons about the value of a healthy work-life balance that I plan to regularly assess throughout my career. Thank you for emphasizing to me the importance of "putting my feet up and thinking about things" and approaching life more calmly. You have truly been a father figure to me and I'm not sure I will ever be able to fully express how much working with you has and will continue to influence my life and career.

I also want to acknowledge the other members of my dissertation committee. Thank you to Dr. Espen Spangenburg for being my go-to person as I designed and conducted these experiments. You have taught me so much about science and how to design experiments. I can always count on you to ask a question that will have me think about science from a different perspective. Thank you to Dr. Eva Chin for the time you

spent with me in the lab, especially when learning about proteomics, and for advice you have given me on science and life. I want to thank Dr. David Mosser for being my Dean's Representative and for being a collaborator on this dissertation work. Your have provided valuable insight with your experiences in cellular and molecular biology. I also want to thank Dr. Steve Prior for your collaboration and guidance over the years. You have helped me to become a better writer and scientist and I am looking forward to learning more from you as my post-doctoral advisor.

Finally, I want to thank my lab mates and fellow graduate students, both past and present. I especially want to thank Dr. Nathan Jenkins for teaching me so much in the lab as a first year doctoral student and for being my "second mentor". Your passion for science is infectious and I look forward to working you more in the future. Thank you to Ryan Sapp for being a great colleague and lab mate. Thank you to the other exercise physiology graduate students especially, Dr. Erik Hanson, Dr. Katie Jackson, Dr. Lindsay Wohlers (Ludlow), Dr. Lisa Guth, Dr. Dapeng Chen, Ana Valencia, Davi Mazala, and Andrew Venezia for being such wonderful friends and individuals to work with. I am so fortunate to have spent this time at the University of Maryland with such incredible people.

Table of Contents

Dedication……………………………………………………………………………….ii
Acknowledgements…………………………………………………………………...iii
List of Tables…………………………………………………………………………vii
List of Figures………………………………………………………………………...viii
Chapter 1: Introduction and Specific Aims……………………………………………...1
    Background………………………………………………………………………...1
    Specific Aim 1……………………………………………………………………..3
    Specific Aim 2……………………………………………………………………..5
    Specific Aim 3……………………………………………………………………..9
    Exploratory Aim…………………………………………………………………..14

Chapter 2: Review of Literature

    Cardiovascular Disease and Endothelial Dysfunction…………….…………17
    History of Circulating Angiogenic Cells and Early Studies……………………18
    Identification of Endothelial Progenitor Cells Versus CACs………………..…19
    Circulating Angiogenic Cells……………………………………………………21
    CAC Isolation by Single Marker………………………………………………...21
        CD34+ CACs…………………………………….……………………21
        CD31+ CACs…………………………………….……………………25
    Discordant Findings in CAC Therapies………………………………………….31
    Role of Oxidative Stress, Nitric Oxide and Inflammation on CAC Function…...33
    Exercise and CAC Function……………………………………………………..38
        Acute Exercise………………………………….……………………..…39
        Exercise Training…………………………….………………………..…40
    Paracrine Functions………………………………………………………………45
    S100A8/S100A9……………………………………………………...…………49
        Structure and Function…………………………………….…………..49
        S100A8/A9 and Exercise……………………………….……………..54
    Summary………………………………………………………………………....57
Chapter 3: Chronic endurance exercise affects paracrine action of CD31+ and CD34+ cells on endothelial tube formation..……………………..…………………………59
    Abstract…………………………………………………………………………..61
    Introduction…………………………………………………………………...…62
    Methods…………………………………………………………………………..65
    Results…………………………………………………………………………....72
    Discussion………………………………………………………………………..77
Chapter 4: Paracrine actions of NSTEMI patients' CD34-/CD31+ CACs impair HUVEC tube formation and are mediated by TLR4………………………...………………98
    Abstract…………………………………………………………...………..100
    Introduction……………………………………………………………………..101
    Methods………………………………………………………………………...…104
    Results…………………………………………………………………….......113
    Discussion……………………………………………………………...……117

Chapter 5: Summary and Future Directions

    Summary………………………………………………………………………..153
    Future Directions……………………………………………………………….155

Literature Cited……………………………………………………………………….161

List of Tables

Table 3.1……………………………………………………………………………86

Table 4.1……………………………………...………………………………...127

Table 4.2…………………………………...…………………………………...128

Table 4.3…………………………………...…………………………………...129

Table S1…………………………………...……………………………………139

List of Figures

Figure 1.1……………………………………………………………………16
Figure 2.1……………………………………………………………………18
Figure 2.2……………………………………………………………………20
Figure 2.3……………………………………………………………………34
Figure 2.4……………………………………………………………………36
Figure 2.5……………………………………………………………………51
Figure 2.6……………………………………………………………………52
Figure 3.1……………………………………………………………………87
Figure 3.2……………………………………………………………………88
Figure 3.3……………………………………………………………………89
Figure 3.4……………………………………………………………………90
Figure 3.5……………………………………………………………………91
Figure 3.6……………………………………………………………………92
Figure 3.7……………………………………………………………………93
Figure 3.8……………………………………………………………………94
Figure 3.9……………………………………………………………………95
Figure 3.10…………………………………………………………………96
Figure 3.11…………………………………………………………………97
Figure 4.1……………………………………………………………………130
Figure 4.2……………………………………………………………………131
Figure 4.3……………………………………………………………………132
Figure 4.4……………………………………………………………………133
Figure 4.5……………………………………………………………………134
Figure 4.6……………………………………………………………………135
Figure 4.7……………………………………………………………………136
Figure 4.8……………………………………………………………………137

Figure 4.9……………………………………………………………………………138

Chapter 1: Background and Specific Aims

Background

Cardiovascular disease (CVD) and the associated dysfunction of the vasculature is the leading cause of death in the developed countries (45). Studies suggest that the repair and maintenance of the vascular endothelium is critically dependent on the number and function of circulating angiogenic cells (CACs) (4, 147, 172). CAC is a broad term for subgroups of peripheral blood mononuclear cells with angiogenic properties that stimulate vascular repair and contribute to neovascularization. CD34 is the most commonly described cell surface marker on CACs that are known for their progenitor cell properties (103). CD31 is a cell surface marker most commonly found in mature endothelial cells, but has also been identified in circulating cells with angiogenic properties (75, 78, 86). Although not as commonly studied as CD34+ CACs for therapeutic neovascularization, CD31+ CACs have been found to exhibit similar angiogenic potential as CD34+ CACs in terms of improving mouse hind-limb ischemia, emphasizing the importance of studying non-progenitor cell lines which may also be involved in angiogenesis (78). Indeed, cells characterized by both markers have previously been found to exhibit angiogenic characteristics *in vitro* (4, 53, 75, 125) and *in vivo* (79, 95, 103).

Originally believed to exert their reparative functions through direct incorporation into the endothelium (4), recent evidence indicates that CACs accomplish their pro-angiogenic effects through paracrine mechanisms. Specifically, CACs are thought to secrete factors that elicit actions on the pre-existing endothelium and/or signal other circulating cells to home to the endothelium (79, 80, 125, 145, 175). Recently, Hynes et al. (2011) demonstrated that the secretome from cultured CACs significantly reduced infarct area in a porcine model of myocardial infarction, suggesting that paracrine factors secreted by CACs may be more important for vascular repair than the cells alone (54). Urbich et al. found that CACs expressed and released a number of different factors that assist with the growth of mature endothelial cells to accelerate revascularization in ischemic tissues (145). This dissertation project will explore the susceptibility of CACs to modulation by physical inactivity or cardiovascular disease.

Physical activity is associated with improvements in CV health, including attenuation of many classic CVD risk factors (65, 98) and improvements in endothelial function (22, 134). Our lab and others have previously shown that endurance exercise training increases CAC number and enhances intracellular redox balance in younger and older adults compared to their sedentary counterparts (63, 66, 94, 146, 147, 166). Hoetzer et al. (2007) found that migratory activity of sedentary individuals' CACs improved by ~50% after 12 weeks of exercise training (50). We previously documented that CACs from young sedentary but otherwise healthy individuals exhibit an unfavorable balance between reactive oxygen species (ROS) and nitric oxide (NO) compared to their endurance-trained counterparts (63, 66). It is generally accepted that CACs from older individuals with CVD exhibit less than optimal function and consequently, cannot contribute properly to vascular repair or neoangiogenesis (35).

Previous studies have examined the effects of endurance exercise habits on CAC function through measures such as migration and adhesion (50, 148), but the effects of physical activity or disease on the paracrine function of CD34+ and CD31+ CACs are less explored. Additionally, the roles of factors secreted from CACs on angiogenesis are not well understood. As such, the purpose of this dissertation is to identify how physical inactivity and cardiovascular disease can affect the paracrine function of CACs and their impact on endothelial tube formation. **Therefore, the overall aim of this dissertation work is to identify key factors secreted by CACs affecting angiogenesis that may be differentially secreted as a function of physical activity status or cardiovascular disease.** Initial investigations assessed the effect of chronic endurance exercise habits on the CAC paracrine factors and how this influences angiogenic tube formation. We also identified secreted factors present in the conditioned medium (CM) of two CAC subtypes and determined whether two identified proteins of interest were differentially secreted as a function of exercise training status. The purpose of this dissertation was to determine the role of the secreted factors from CVD patients' CACs on angiogenic tube formation and to mechanistically determine whether certain proteins are contributing to the dysfunctional CAC properties observed in CVD patients.

Specific Aim #1: To determine whether there is a differential response of paracrine effects of CD34+ and CD34-/CD31+ CACs on angiogenic tube formation as a result of chronic endurance exercise habits in younger men and women.

The purpose of this study was to determine whether there is a differential response in paracrine effects of CD34+ and CD34-/CD31+ CACs on angiogenic tube formation as a result of chronic endurance exercise habits in younger men and women. Additionally, we aimed to identify differences in CAC intracellular redox pathways that may be eliciting angiogenic or anti-angiogenic actions. In this study we demonstrated that conditioned media (CM) generated from inactive subjects' CD34+ and CD34-/CD31+

CACs depressed human umbilical vein endothelial cell (HUVEC) tube-formation. Furthermore, there appears to be a graded response as a function of habitual exercise levels with CM from inactive individuals contributing to lower HUVEC tube formation compared to their active and endurance-trained counterparts. These data strongly suggest that endurance exercise training-mediated alterations in CAC paracrine activity influence angiogenesis. Other studies have demonstrated the role of paracrine signaling of CACs in disease states (54, 125, 174), but our data provide further support for a paracrine role for CD34+ and CD34-/CD31+ CACs on angiogenesis in younger, healthy individuals. Importantly, to our knowledge, this is the first report of a differential paracrine role in CD34+ and CD34-/CD31+ CACs on HUVEC-based tube formation as a function of chronic physical activity habits. These data emphasize the negative impact that a sedentary lifestyle can have on cellular biological processes even in younger, otherwise healthy adults before other, classic risk factors for CVD are present. As previous research in our lab would support (63), we hypothesized that differences in intracellular ROS and NO concentrations as a result of exercise-training status may, at least partially, explain the observed differences in paracrine function in both cells types. Surprisingly, our data suggest that intracellular ROS and NO may not play a major role in regulating the differential paracrine responses observed. There were no significant effects of training status on intracellular NO or ROS levels or in mRNA expression for eNOS as well as $p47^{phox}$ and $gp91^{phox}$ for either cell type. As such, we began investigating potential differences in secreted factors as a function of exercise training status that may contribute to the differential effects of tube formation caused by CAC conditioned media. Specific aims #2 and #3 of this dissertation focus on the identification of novel proteins secreted by CACs and the effects of habitual physical activity and CVD on the secretion of these proteins.

Specific Aim #2: To identify proteins present in the CM of CD34+ and CD34-/CD31+ CACs and to determine whether these proteins are differentially expressed as a function of chronic exercise training status.

In an attempt to further investigate potential contributors to our observed differences in tube formation, we used mass spectrometry and identified two members of the S100 family of calcium-modulated proteins, S100A8 and S100A9, in the secretome of both CD34+ and CD34-/CD31+ CACs. S100A8 and S100A9 proteins are abundantly expressed in both myeloid and vascular cells (5) and are most commonly known for their role in regulating inflammatory processes. These proteins can function independently as homodimers or as a S100A8/A9 heterodimer complex. Several studies have linked systemic concentrations of the S100A8/A9 heterodimer complex to CVDs (5) with higher plasma levels predicting future CV events in middle-aged healthy individuals (17, 18). As S100A8 and S100A9 have established ties to CVD and inflammation, we decided to focus the investigations for aims 2 and 3 on the role of these proteins in angiogenesis. Upon identification of these proteins in the conditioned media of both CD34+ and CD34-/CD31+ CACs, we confirmed that both CAC sub-types expressed the mRNA for both of these proteins, although no differences in mRNA expression were found as a function of physical activity habits. As our observed differences in tube formation appeared to be a graded effect, we focused on the two extreme groups, endurance-trained and inactive, for protein analyses in the conditioned media. The purpose of this investigation was to determine if levels of S100A8 and S100A9 in CD34+ and CD34-/CD31+ conditioned media were differentially expressed as a function of chronic exercise training status. In this study, we did not observe an effect of training status on S100A8 or S100A9 protein expression in CD34+ CM that might explain the differences in CM-induced angiogenesis. However, we found that there was a 103% more S100A9 content in the CD34-/CD31+ CACs from inactive subjects compared to their endurance-trained counterparts. These findings are the first, to our knowledge, to demonstrate differential secretion of S100A9 by CD34-/CD31+ CACs as a function of an individual's exercise-training status. Although not statistically significant, S100A8 content was approximately twice as high in the CD34-/CD31+ CM from inactive subjects compared to endurance-trained subjects. Given the biology of the S100A8/A9 complex, it is possible that the S100A8 findings in CD34-/CD31+ CM paired with the significant differences in S100A9 may be physiologically relevant. However, future studies assessing the impact of the complex as a whole and functions of different proportions of S100A8 to S100A9 are necessary to confirm these speculations. These results suggest that increased concentrations of S100A8 and S100A9 secreted by CD34-/CD31+ CACs may contribute to lower tube-formation in inactive individuals.

In an effort to estimate approximately how much of each protein was in the CM to which the HUVECs were exposed to in specific aim #1, we used recombinant human S100A8/S100A9 and created a standard curve using known concentrations found in the literature and compared those to the concentrations found in a subset of samples from this study. We found that the CM from both cell types contained substantially less S100A8 and S100A9 than other investigations assessing the *in vitro* effects of these proteins (5, 92, 151). Thus, CM content in this study is on the low end of the established *in vitro* range of S100A8 or S100A9 concentrations. Defining the concentrations of S100A8 or A9 is important since previous publications have shown concentration dependent effects on angiogenic function (92, 151). In addition, both proteins are able to activate signaling in a homodimer, heterodimer or heterotetramer form (152), thus adding more complexity to their mechanism of action. Importantly, when the ratio of S100A8/S100A9 was calculated in the CM there was a greater proportion of S100A8 to S100A9 in the CD34-/CD31+ CM compared to that of the CD34+ CM (2.343 vs. 0.513, respectively). These results suggest that CM from the different cell types are fundamentally different from one another even though both showed similar effects on the HUVEC tube formation. The differences in the ratios of these proteins in the CM of the two different cell types may provide one explanation for the observed differences in these proteins as a function of training status in the CD34-/CD31+ CM but not in the CD34+ CM. This also suggests that other secreted factors may also be participating in the observed differences in CD34+ CM-mediated HUVEC tube formation as a function of training status.

To confirm the direct effects of S100A8 and S100A9 on HUVEC tube formation, the concentrations and proportions estimated in the CM of the inactive subjects' CD34-/CD31+ CACs were used in an angiogenesis assay. As estimated, 7.18 μg/mL of recombinant human S100A8 (ProSpec Bio) and 3.06 μg/mL of recombinant human S100A9 (Life Technologies) were added to a HUVEC-based tube-like formation assay and compared to the positive control prepared with EBM-2 and vehicle control. In these experiments, each condition was assessed in samples collected from six independent cell culture wells from multiple culture plates collected on different days and on cells from the same passage number. Compared to the basal control, addition of S100A8 and S100A9 resulted in an average of 18% lower tube length and 28% lower complexity. These data indicate the direct effects of S100A8 and S100A9 on HUVEC tube-formation and support our earlier findings suggesting that differences in S100A8/A9 present in the CM may be responsible for the differential tube formation observed as a function of exercise-training status.

Based on the results of specific aims #1 and #2, we concluded that a) there is a differential paracrine role of CD34+ and CD34-/CD31+ CACs on HUVEC-based tube formation as a function of chronic physical activity habits, b) pro-inflammatory S100A8 and S100A9 secreted by CD34-/CD31+ CACs is twice as high in the CM of inactive individuals compared to their endurance-trained counterparts, and that c) recombinant S100A8 and S100A9 added in concentrations estimated to be present in the CM of inactive CD34-/CD31+ CAC significantly reduce HUVEC tube-formation. To summarize the dissertation work completed thus far, increases in secreted pro-inflammatory factors S100A8 and S100A9 in inactive individuals appear to contribute to the reduced HUVEC tube-formation associated with CM from CD34-/CD31+ CACs. If a sedentary lifestyle promotes secretion of inflammatory factors, one would assume that this effect would then be exacerbated with age and progression of CVD. As such, the final aim of this dissertation will focus on studying the paracrine effects of CACs from CVD patients.

Specific Aim #3: To determine if inhibition of S100A8/A9 receptors will improve the depressed tube formation of HUVECs cultured with CD31+ CM from CVD patients compared to endurance-trained individuals.

Recently, various sub-fractions of CACs have been used in autologous intra-coronary cell transplants in attempt to to treat ischemic CVD and improve vascularization in humans (95, 120). Losordo et al. reported decreased angina frequency and improved exercise tolerance in patients with refractory angina in a clinical trial in which purified autologous CACs were injected into the injured area (95). While promising, the broader spectra of clinical trials in this area have reported only moderate success. As mentioned earlier, it is well established that individuals with CVD have fewer numbers and lower functioning CACs than healthy individuals (35, 44, 142). Thus, some of the variability in the literature can potentially be attributed to the autologous nature of the cell transplants, in which presumably lower functioning cells from a diseased patient are used to treat the same patient. This would hamper the use of CACs for autologous treatment in CVD patients, who are in the greatest need for such therapies. As such, it is critical to understand more about how CACs from CVD patients function and what tools we can use to improve their function to enhance the therapeutic use of these cells. As both the literature (79, 126) and our lab (87) have demonstrated the importance of CAC paracrine actions on endothelial cells, the focus of this final dissertation project will be on understanding the paracrine role of CACs from CVD patients.

Hypothesis I: Culture of HUVECs with CM from CD34-/CD31+ CACs of CVD patients will result in lower HUVEC tube formation compared to endurance-trained subjects and this will be associated with higher levels of S100A8 and S100A9 in the CD34-/CD31+ CM of CVD patients.

This hypothesis was tested by isolating and culturing CD34-/CD31+ CACs from CVD patients and endurance-trained subjects to allow for secreted factors to be released into the culture medium. CM derived from cultured CACs will then be used in a HUVEC-based tube formation assay as we have done previously in specific aim #1 to determine if secreted factors from CACs of each group will contribute to a differential endothelial tube formation. Endothelial tube formation with the commonly used HUVEC line is a well accepted functional assay and the use of cell-free CM will enable the measure the effects of secreted factors from CD34-/CD31+ CACs of CVD patients compared to a model of optimal CAC function, endurance-trained subjects. A proteomics approach on a subset of samples from each group is proposed to confirm our previous findings and to identify other potential proteins in the CD34-/CD31+ CM that are differentially present or expressed between CVD patients and younger endurance athletes. Additionally, as our earlier studies indicate, S100A8 and S100A9 are likely candidates to explain the lower tube formation hypothesized for the CVD patients' CD34-/CD31+ CM. Thus, Western blots examining the differences in levels of these proteins in the conditioned media of CD34-/CD31+ CACs from each subject group will be used as was done in specific aim #2.

By investigating the pro-inflammatory S100A8 and S100A9 proteins in specific aim #2 we have focused on potential factors that may contribute to the dysfunction in CACs of younger, inactive adults as a means of identifying potential early risk factors for the development of endothelial dysfunction and CVD. A sedentary lifestyle is associated with future CVD risk independently and through diseases such as obesity and Type II Diabetes, and it is possible that differences in the secretion of these proteins in sedentary but otherwise young and healthy individuals will become more exacerbated with age and progression of CVD in the absence of lifestyle changes. As such, our previous findings, if used as a predictor of future CVD risk, might be extrapolated to explain some of the dysfunctional properties of CACs from CVD patients.

As mentioned, S100A8 and S100A9 plasma concentrations positively correlate with CVD risk (17). Thus, the presence of these proteins in our CM indicates a potential role of CAC paracrine factors in the pathological setting of cardiovascular disease. However, the direct effects of secreted S100A8 and S100A9 on the endothelial microenvironment is still not completely understood. Investigations into these pathways and the mechanisms through which S100A8/A9 secreted by CACs function in CVD patients are important issues for future research in this area. As such, the next purpose within specific aim #3 is to mechanistically determine how S100A8 and S100A9 secreted by CD34-/CD31+ CACs from CVD patients are contributing to depressed endothelial tube formation. The importance of studying CVD patients, the same individuals who are, in theory, candidates for such autologous CAC treatments, is clear. Additionally, comparing them to endurance trained athletes is a practical step based on the findings of our lab indicating these individuals serve as a model for optimal CAC function. A number of recent studies have begun to focus on therapies to enhance the function of CACs (13, 70, 89). The results of many of these studies are promising in both *in vitro* and *in vivo* animal models (13, 58). However, what “improved” function means for relevant physiological and clinical outcomes is yet to be determined. Our lab has shown that

CACs from endurance-trained younger and older athletes are of higher number and function compared to their sedentary peers (63, 87, 166). We have previously found that CACs from young sedentary but otherwise healthy individuals exhibit an unfavorable intracellular redox balance compared to endurance-trained individuals (63). In addition, results from many other laboratories support the role of aerobic exercise in enhancing the function of CACs (63, 94, 146-148). As such, we believe in using young, endurance-trained athletes as a model of "optimal" CAC function as a comparison and ultimate goal when treating CACs to enhance their function prior to cell therapy.

Hypothesis II: Inhibition of receptors for S100A8 and S100A9 on HUVECs will prevent S100A8 and S100A9 from binding and consequently rescue HUVEC tube formation when cultured with CM from CVD patients to levels similar to those observed when HUVECs are cultured with CM from endurance-trained subjects.

Research investigating the role of S100A8 and S100A9 in CVD has found that these proteins can affect endothelial function in a number of ways. *In vivo*, it has been found that S100A8/A9 are involved in leukocyte recruitment during the response to vascular injury, promoting atherosclerotic lesions (18). *In vitro* studies have found that these proteins can decrease endothelial monolayer integrity (151, 162), and increase adhesion molecules and other pro-inflammatory factors on HUVEC cells (151). However, the effects of these proteins in the concentrations secreted by CACs of individuals of different health and disease statuses are still not completely understood. As such, the final aim of this dissertation is designed to mechanistically alter the ability of S100A8 and S100A9 present in the CD34-/CD31+ CM of our CVD patients and endurance athletes to bind to and act on HUVEC cells and to assess the level of HUVEC tube formation under these conditions.

Two major well-known receptors for S100A8/A9 are the receptor for advanced glycation end products (RAGE) (11)and Toll-like receptor-4 (TLR4) (26, 155), with S100A8 having a preference for TLR4 and S100A9 preferring RAGE (162). When the S100A8 and S100A9 proteins bind to RAGE and TLR4, they modulate calcium signaling and cytoskeletal reorganization (18, 48, 154) through p38 and ERK1/2 signal pathways downstream of these receptors (162). One investigation into these signaling pathways in HUVEC cells found that S100A8/A9 caused endothelial barrier dysfunction increasing HUVEC monolayer permeability through RAGE and TLR4/p38/ERK1/2 signaling (162). We are interested in the effects of the levels and proportions of S100A8 and S100A9 present in the secretome of CD34-/CD31+ CACs of patients with CVD on HUVEC-based angiogenic tube formation compared to those present in the secretome of endurance trained individual’s CACs. Using an antagonist for RAGE and TLR4 signaling inhibitor both individually and in combination to prevent the downstream effects of S100A8/A9 binding, HUVECs will be pre-treated before use in the tube formation assay to determine how inhibition of these receptors and thus, the ability of these proteins to bind to and act on HUVECs through their major receptors will affect the tube formation capacity. It is expected that tube formation using the CM from CVD patients will result in lower HUVEC tube formation and that pretreatment with these receptor antagonists will allow tube formation to be restored to a level similar to that observed when using CM from endurance-trained athletes.

As the differences in levels of S100A8 and S100A9 as a function of training status in specific aim #2 were observed only in CD34-/CD31+ CACs, specific aim #3 will focus on the differences in S100A8 and S100A9 in the conditioned media of just CD34-/CD31+ CACs. CD34-/CD31+ CACs have been found to exhibit similar angiogenic potential as the more commonly studied progenitor-type CACs in terms of improving mouse hind-limb ischemia (78). Indeed, cells characterized by this marker have been previously found to exhibit angiogenic characteristics both *in vitro* (75, 84) and *in vivo* (79). Given the proven angiogenic capacity of this cell type, coupled with the results of specific aim #2, we have decided to focus on this one cell type for the final aim of this dissertation work. Additionally, there are significantly more CD34-/CD31+ CACs present in peripheral blood compared to CD34+ CACs, which have even more reduced circulating numbers in individuals with CVD (35, 103). As such, a more mechanistic project can be completed using the CD34-/CD31+ CAC subtype due to their greater numbers, and perhaps more biologically significant in terms of their paracrine functions and effects on angiogenesis based on the earlier findings in specific aim #1 and #2.

Exploratory Hypothesis: S100A8 and S100A9 present in the CD34-/CD31+ CM of CVD patients act through p38/ERK/NFκB signaling pathways of endothelial cells and inhibition of the receptors for these proteins on HUVECs will reduce expression of these proteins.

Protein detection in frozen CM has been done successfully in our lab in the past (87). However, frozen CM in has not been found viable in cell culture experiments in our hands. As such, in an effort to collect additional data for future experiments in which we begin to explore the signaling pathways through which S100A8/A9 are acting on the

HUVEC cells, we plan to use fresh CM to perform cell culture experiments as an exploratory aim. Literature indicates that S100A8 and S100A9 act through TLR4 and RAGE and signal downstream MAP kinases that increase endothelial cell permeability and cytoskeletal structure of HUVECs (18, 162). This exploratory aim will examine whether effects of the CVD patient's CD34-/CD31+ CM may also be occurring through this same pathway to reduce HUVEC function. The expression of different proteins downstream of each receptor on HUVEC cells exposed to CM from each subject population with or without HUVEC pretreatment with a RAGE antagonist and/or TLR4 inhibitor will be examined. Phosphorylation of the MAP kinases, p38 and ERK1/2, and expression of NFκB using western blot to determine if a) conditioned media from CVD patients up-regulates expression of each of these proteins to a greater degree compared to endurance-trained subjects, and b) pretreatment of HUVECs with a RAGE antagonist and/or TLR4 inhibitor will down-regulate expression of p38, ERK1/2, and NFκB to levels closer to those expected with HUVEC exposure to endurance-trained subjects' CD34-/CD31+ CM are proposed to be measured.

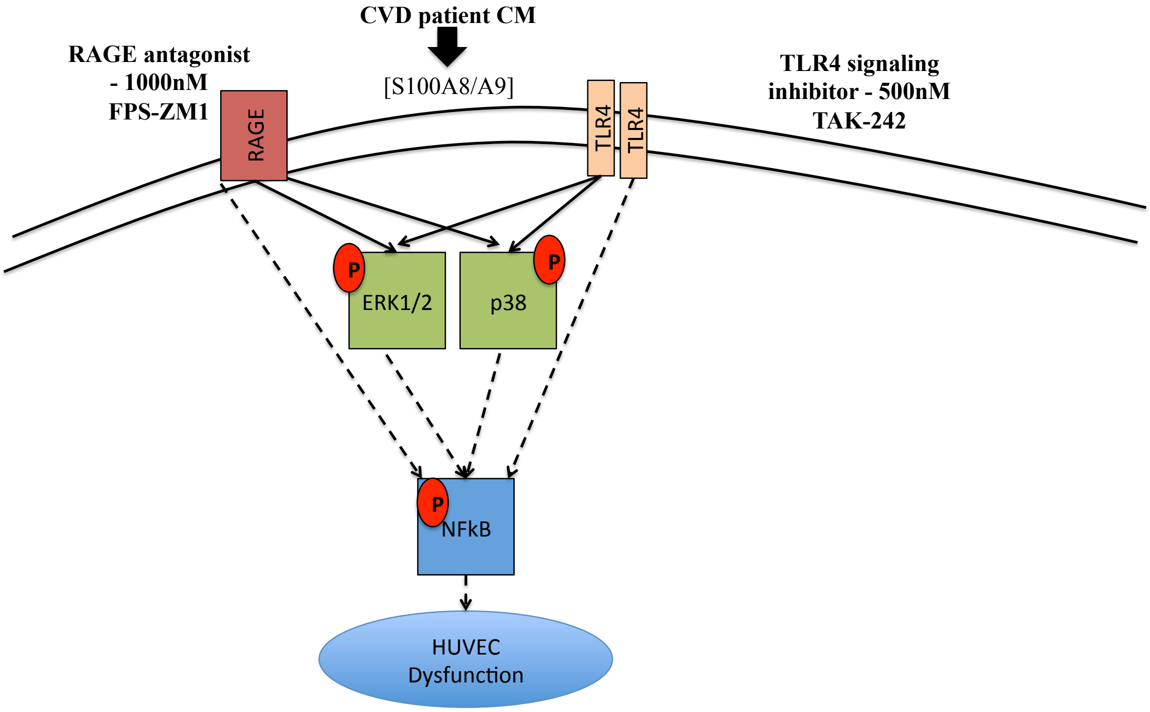

CVD patient CM
[S100A8/A9]
RAGE antagonist
- 1000nM
FPS-ZM1
RAGE
TLR4
TLR4
TLR4 signaling
inhibitor - 500nM
TAK-242
P
ERK1/2
P
p38
P
NFkB
HUVEC
Dysfunction

Figure 1.1 Downstream signaling for two major receptors for S100A8/A9 and proposed targets of measurement for exploratory aim to assess potential signaling pathways involved in HUVEC dysfunction when exposed to factors secreted from CVD patients' CACs. Figure adapted from Wang et al. (2014) (162)

Chapter 2: Review of Literature

The purpose of this literature review is to examine the research investigating circulating angiogenic cell function, specifically regarding paracrine actions of CACs, and the role that exercise and cardiovascular disease play in modifying these functions.

Cardiovascular Disease and Endothelial Dysfunction

Cardiovascular disease (CVD) is the leading cause of death in developed countries around the world (45). It is estimated that by 2030, over 40% of the US population will have some form of CVD with direct medical costs tripling as a result (45). Advancing age, physical inactivity, obesity, dyslipidemia, diabetes, hypertension and smoking are just some of the traditional risk factors involved in the development of CVD. Each of these factors, independently or coupled with others, contributes to impaired endothelial function, which is one of the first steps in the development of CVD and often precedes clinical cardiac events (22, 134). Over time, an imbalance occurs between inflammatory, oxidative stress, and vasodilatory factors that leads to vascular endothelial dysfunction, which then contributes to the increased risk of developing CVD (134) (Figure 2.1). However, traditional CV risk factors, such as those listed above, explain only ~50% of CVD events, (12, 112) providing a need to investigate other novel CVD risk factors that help further our ability to prevent and treat CVD and the associated endothelial dysfunction.

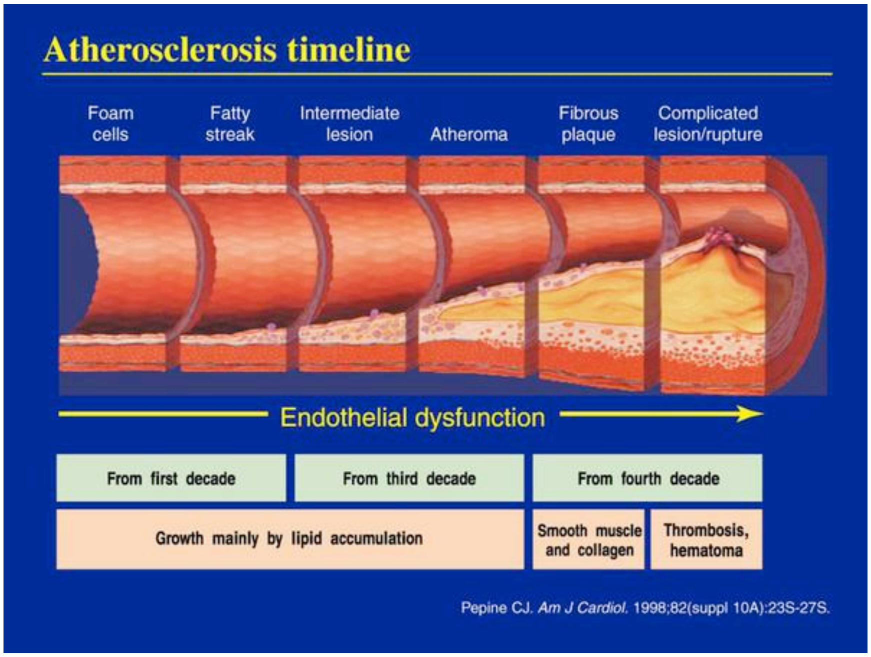

Atherosclerosis timeline
Foam cells
Fatty streak
Intermediate lesion
Atheroma
Fibrous plaque
Complicated lesion/rupture
Endothelial dysfunction
From first decade
From third decade
From fourth decade
Growth mainly by lipid accumulation
Smooth muscle and collagen
Thrombosis, hematoma
Pepine CJ. Am J Cardiol. 1998;82(suppl 10A):23S-27S.

Figure 2.1 Progression of atherosclerosis over time and associated endothelial dysfunction (119)

History of Circulating Angiogenic Cells and Early Studies

In 1997, Asahara and colleagues first isolated putative endothelial progenitor cells (EPCs) from human blood using immunomagnetic separation methods (4). These cells were able to differentiate into mature endothelial cells *in vitro* and also were incorporated into sites of vascular damage in an *in vivo* animal model of hind limb ischemia (4). This was the first demonstration of the efficacy of adult circulating cells with angiogenic potential as a potential therapy for treating CVD and, since then, a vast amount of research has studied circulating cells that can aid in therapeutic angiogenesis. Asahara et al., (1997) used two cell surface markers, CD34+ and vascular endothelial growth factor receptor 2 (VEGFR2+ or KDR+) to identify these angiogenic cells, due to the presence of these markers on early hematopoietic cells and mature endothelial cells but not differentiated hematopoietic cells (4). Since this study was published, the field of "EPCs" has grown and the definition of a true EPC has become widely debated (171). This has lead to the identification of a number of other circulating cell types that do not fit the strict definition of EPC (29, 171). Thus, the term circulating angiogenic cell (CAC) has been adopted as a global term to represent all cell types found in the circulation or cultured from circulating cells that have angiogenic properties that are associated with endothelial repair and maintenance.

Identification of Endothelial Progenitor Cells Versus CACs

EPCs are generally characterized as "early" EPCs (eEPCs) and "late" or "outgrowth" EPCs (OEC). Both can be derived from culturing human peripheral blood mononuclear cells (PBMCs) on fibronectin or gelatin after removal of nonadherent cells on day 2. Early EPCs appear after 4-10 days of culture and express CD45 and typical myeloid markers, such as CD14. They have been found to have high paracrine activity, and some have found that they can incorporate into capillaries (29, 53, 69, 149). Using the same culture conditions as described above, OEC generally appear after two or more weeks of culture and have been found to be molecularly distinct from eEPCs (108). OECs exhibit lower paracrine activity and incorporate well into capillaries (29, 53, 69, 149). Although both types of EPCs have been associated with functional improvements in the treatment of hindlimb ischemia and acute myocardial infarction, the molecular phenotype of eEPCs is more closely linked to monocytes while OECs are more committed to an endothelial lineage (108).

Others have found that culturing PBMCs on collagen for 2-3 weeks results in a cell type that is believed to be the "true" EPC because they lack CD45 and CD14 and most closely resemble endothelial cells (57, 171). These cells, termed endothelial colony-forming cells (ECFCs), have high proliferation capacity and exhibit *in vivo* vessel growth on matrigel. However, these cells lack the progenitor cell marker and some speculate that they may be either derived from the vascular wall rather than bone marrow or a product of their culture environment as these "true" EPCs are not detected in circulation (29, 53).

Hill et al. developed another method of identifying "EPCs" through culture of the non-adherent cells after PBMCs were cultured for 48 hrs. Using this method, colony-forming units (CFU-ECs) appear around 5 days and are comprised of a heterogenic population of cells including cells with progenitor, endothelial, myeloid and T-cell markers (29, 35). Due to the heterogenic nature of CFU-ECs and their similarity to monocytes, it has been generally agreed that these cells should not be regarded as EPCs, but rather, have been given the name "circulating angiogenic cell" (CAC) (29).

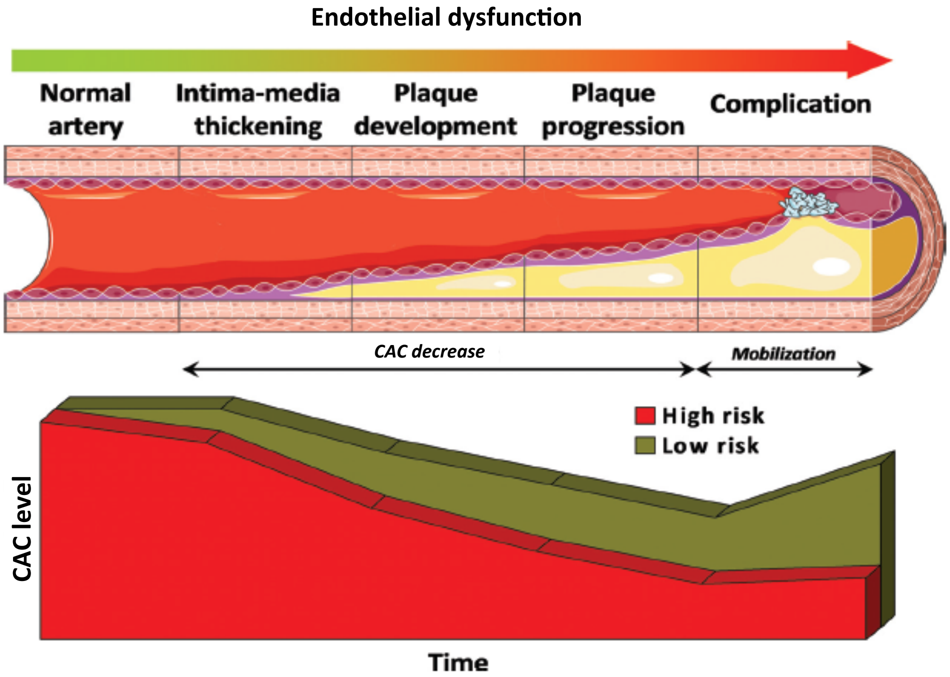

Endothelial dysfunction
Normal artery
Intima-media thickening
Plaque development
Plaque progression
Complication
CAC decrease
Mobilization
🟥 High risk
🟩 Low risk
CAC level
Time

Figure 2.2 Association between CAC number, endothelial dysfunction and progression of cardiovascular disease. Figure derived from Fadini et al. (29)

Circulating Angiogenic Cells

Direct comparison of results from various studies has proven difficult due to the different classifications and culture conditions for “EPCs”. As no single cell marker to date has been unanimously accepted as identifying “true” EPCs, many of these cells, including the CFU-ECs, eEPCs, and cells isolated by single or multiple cell markers are now being classified under the umbrella term CAC. As a whole, it is generally accepted that CACs are involved in the repair and maintenance of the vascular endothelium, with the number and function of certain CACs being inversely related to CVD risk (7, 8, 46) (Figure 2.2).

Selection of cells using a single or multiple cell surface antigens has been used to enrich culture environments (4, 126) in order to study the function and properties of specific purified cell populations freshly isolated from peripheral blood from humans of different health statuses or in response to an intervention (63, 95, 165). Some of the more commonly studied cell markers include CD34+, CD133+, VEGFR2+ and CD31+. As all of the above listed cell types have been found to exhibit angiogenic properties (29, 35, 75, 78, 79, 95, 103), for purposes of this review, the broad term CAC will be used with specific cell markers listed where appropriate.

CAC Isolation by Single Marker

CD34+ CACs

CD34 is the most commonly described cell surface marker on CACs that are known for their progenitor cell properties (103). This cell type rose in popularity as a circulating cell with angiogenic potential due to the presence of the CD34 antigen on both progenitor cells and fully differentiated vascular endothelial cells (103). As such, it was believed that these circulating cells could adopt an endothelial phenotype and contribute to the development of new blood vessels in response to ischemic damage resulting from an acute CV event (95, 103). Although the complete cellular functions of the CD34 protein are not completely understood, there is support for its role in promoting proliferation of hematopoietic progenitor cells from both the blood and bone marrow (16). The basal density of CD34+ cells in circulation without the use of mobilizing agents is relatively low (~1% of total mononuclear cells (MNCs), with advanced age and chronic CVD contributing to a decrease in both number and functional properties of these cells (103).

Although CD34 is arguably the most commonly studied cell surface marker, it is usually studied in combination with endothelial markers (i.e. VEGFR2+) and/or other progenitor cell markers (CD133+). There have been, however, a few groups who have investigated CD34+ through immunomagnetic isolation of just the single marker. In 2006, Kawamoto et al. provided one of the first examples of purified mobilized human peripheral blood CD34+ cells as an effective treatment for myocardial ischemia compared to total MNCs (72). They found that treatment with CD34+ CACs improved capillary density, reduced percent fibrosis, and better preserved echocardiogram regional wall motion score compared to treatment with high and low doses of MNCs in rats after experimental myocardial infarction (MI) (72). Other studies found that CD34+ CACs were more effective in healing and improving vessel growth after hindlimb ischemia in mice compared to CD14+ monocytes (6). Due to some promising results in these earlier animal studies, autologous cell transplants have been tested in human clinical trials for various ischemic diseases. A Phase I/II randomized double-blinded trial published in

2007 provided evidence for the safety and feasibility of autologous CD34+ cell therapy for treatment of angina (97). Subsequent clinical trials by the same lab examined the bioactivity of these autologous cell therapies in patients with refractory angina and found improvements in angina frequency and exercise tolerance (95) with a phase III clinical trial currently underway (106).

Further pre-clinical trials in Lorsordo's lab have also begun exploring the feasibility for autologous intramuscular transplantation of CD34+ cells for the treatment of individuals with critical limb ischemia. An initial randomized placebo controlled pilot study found favorable results with lower amputation rates (22% versus 75% of controls) in the CD34+ CAC treated group with critical limb ischemia one-year post treatment (96). Later phase II clinical trials found improvements in a number of physiological parameters including skin perfusion pressure, pain free walking distance and transcutaneous partial oxygen pressure in patients just 1-2 weeks after treatment, but plateauing after about 9 months (37).

The mechanism of action when CD34+ cells are used for therapeutic neovascularization is still not completely understood. Early studies show evidence of differentiation of CD34+ cells into endothelial cells and cardiomyocytes after transplantation into nude mice following coronary artery ligation (61). However, other studies using non-human primate autologous CD34+ cells tagged with green fluorescent protein (GFP) found very few transplanted cells incorporated into the vasculature or in cardiomyocytes (173). Rather, these authors found that cultured CD34+ cells secrete angiogenic cytokines that promote angiogenesis in endogenous endothelial cells (173). Similarly, Wang et al., found that functional improvements in the hearts of severe combined immunodeficiency (SCID) mice after experimental MI were due to improvements in angiogenesis and/or paracrine effects, but not a result of myogenesis (160). More recent studies have since supported this notion that CD34+ CACs function mainly through paracrine effects (145, 168). Indeed, Sahoo et al. (2011) found that the angiogenic effects of CD34+ CACs are mediated by secreted exosomes (125). They found that conditioned media generated from CD34+ CACs promoted endothelial cell viability, proliferation and tube formation (125) and that CD34+ exosome-only treatment replicated the results observed in these *in vitro* experiments. *In vivo* treatment using CD34+ derived exosomes successfully stimulated angiogenesis using matrigel plug and corneal assays (125). Interestingly, when CD34+ conditioned media was depleted of exosomes or when exosomes from CD34+ -depleted MNCs were used these angiogenic activities were no longer evident, emphasizing the effects of paracrine factors, namely exosomes, in CD34+ CAC angiogenic actions.

The survival of CACs after transplantation is another topic that is still under investigation. Using bioluminescence imaging, Wang et al. (2010) found that transplanted CD34+ cells survived in the hearts of SCID mice after experimental MI for more than one year after cell therapy (160). Other studies have shown that engraftment of transplanted CD34+ cells in human patients with MI is only ~5% 1 hour following injection and that these cells are retained preferentially in the peri- infarct zone of the heart (115). A larger, more recent study of 110 patients with dilated cardiomyopathy examined the effects of CD34+ transplantation after 5 years. Two hours after CAC treatment, engraftment was estimated to be ~7% and this decreased to 5% at 18 hours following injection as indicated through ^{99m}Tv-hexamethylpropylene-amine oxyme labeling. These investigators found that long-term survival, ventricular function, and exercise tolerance were greater in the group that received the cell therapy compared to controls at five years after cell transplantation (158). Improvements in left ventricular ejection fraction were greater in patients with better myocardial homing of injected CACs after 3 and 12 months. Thus, it appears that homing of CACs to the area of damage affects some outcome measures but that the long-term effects of CD34+ therapy may persist even if the cells have not engrafted into the endothelium.

CD31+ CACs

CD31, also known as Platelet endothelial cell adhesion molecule-1 (PECAM-1), is a cell surface marker most commonly found on mature endothelial cells, but it has also been identified on hematopietic cells (64, 79, 85), monocytes, granulocytes, platelets (167) and T cells (53). On endothelial cells, CD31 is involved in the maintenance of a vascular permeability barrier, regulation of transendothelial migration of monocytes and neutrophils, and in angiogenesis (78). Although not as commonly studied as CD34+ CACs for therapeutic neovascularization, CD31+ CACs have been found to exhibit a similar angiogenic potential as CD34+ CACs in terms of improving mouse hind-limb ischemia, emphasizing the importance of studying non-progenitor cell lines which may also be involved in angiogenesis (78). Selected human CD31+ cells have been found to express higher levels of angiogenic genes compared to the CD31 depleted fraction (75, 79). When injected into a mouse ischemic hindlimb, CD31+ CACs displayed high adhesion and vasculogenic capacity and augment blood perfusion preventing limb loss (75, 79).

Bone marrow-derived CD31+ cells have been identified as a highly angiogenic subgroup of mononuclear cells (75, 79). CD31+ cells exhibit an endothelial phenotype *in vitro* (79). Indeed, there has been at least one report of CD31+ CACs spontaneously forming tube-like formations *in vitro* that were positive for lectin and took up acetylated LDL, a characteristic similar to endothelial cells (79). Additionally, other studies have found that incorporation of CD31+ cells into the existing vasculature was significant, although transplanted cells accounted for only ~3% of the functional endothelial cells present in a mouse hindlimb ischemia model, despite an observed reversal in ischemia and 1.3 fold increase in capillary density (75). These results suggest that other mechanisms, such as paracrine function, are playing a more dominant role in the reparative activities of CD31+ cells. Indeed, Kim et al. has identified an upregulation of a number of angiogenic, anti-apoptotic and chemotactic factors in the ischemic limbs 7 days after transplantation of CD31+ cells, providing support for paracrine activity as a major method of action in CD31+ CACs (75, 79).

The cell surface markers present along with CD31+ on CACs may play a large role in determining their physiological function. Many studies have investigated angiogenic T-cells, which contain the marker CD31+ due to their roles in inflammation. Hur et al. (2007) found that CD31+/CD3+ T cells, but not CD31-/CD3+ cells constitute the central cluster of the CFU colonies and play an important role in vessel formation mainly through paracrine actions (52). In the clinical setting, these authors also found that the number of CD31+/CD3+ cells found in peripheral blood was inversely related to age and number of CV risk factors (52). Indeed, as with other CAC subtypes, CD31+/CD3+ cells are considered novel risk factors for CVD. Although the direct relationship between

CD31+/CD3+ CACs and clinical presentation of CVD has not been well explored, to date, there is evidence of increased number of CD31+/CD3+ CACs in patients with acute coronary syndrome compared to healthy controls (68). This difference was no longer evident 6 months after percutaneous coronary intervention suggesting that acute coronary events promote mobilization of CD31+/CD3+ CACs (68). Another study investigating healthy men between the ages of 21-70 years found that CVD risk and *in vivo* endothelial function was not associated with CD31+ T cell number. However, CD31+ T cell migratory capacity was positively correlated with forearm blood flow response to acetylcholine (Ach) and inversely related to Framingham Risk Score (164). Another study assessed the effects of age on CD31+ T cell number and function. Aging is associated with increased risk of CVD and progression of atherosclerosis due, in part, to a diminished endogenous vascular endothelial repair capacity. Kushner et al. (2010) found that older age (56-75 yrs) is associated with lower CD31+ cell number, reduced migratory capacity, shorter telomere length and reduced telomerase activity, and an increase in susceptibility to apoptosis compared to younger men (20-35 yrs) (86). Interestingly, these authors observed no differences in CD31+ number in middle-aged (36-55 yrs) versus younger men, suggesting that in healthy men, CD31+ number is preserved through middle age with the decline in cell number occurs after ~55 years of age (86). These studies are important as they demonstrate the critical role of CD31+ CACs as a novel CV biomarker in both healthy populations and individuals with CVD and emphasize the role of not just CD31+ cell number but also the function of these cells in contributing to the repair and maintenance of the endothelium.

Importantly, expression of CD31 on leukocytes controls T cell activation and lymphocyte hyperactivity, which plays an important role in the regulation and stabilization of atherosclerotic plaques (34). Loss of CD31 regulatory signaling in T cells has been shown to promote atherlosclerosis (15). Flego et al (2014) found that CD31 expression on T cells from patients with acute coronary syndrome was reduced compared to patients with stable angina, leading to reduced inhibitory activity in lymphocytes and T cell dysregulation (34). These findings suggest that CD31 expression is critical for the regulation of the immune response associated with atherosclerosis. Hur et al (2007) demonstrated that CD31+ T cells are able to form better tubules, have greater transendothelial migration, and have a greater ability to promote endothelial cell proliferation and function *in vitro* compared with other T cells subsets emphasizing the critical role of CD31+ CACs in angiogenesis (52). Indeed, others have found that CD31+/CD3+ CACs have better migratory capacity in response to SDF-1α and enhanced secretion of the angiogenic cytokines G-CSF, IL-8 and MMP-9, compared to CD31-/CD3+ CACs, which they conclude may be contributing to the enhanced vasculogenic properties as compared to CACs lacking CD31 (84). The enhanced migratory capacity of cells positive for CD31 may be attributed to greater surface expression of CXCR4 (98% compared to 38% in CD31- CACs), which is the receptor for SDF-1α (53).

Monocytes (CD34-/CD14+) cultured under angiogenic conditions have been found to take on an endothelial phenotype and form cord- and tube-like structures in *in vitro* (132). Importantly, CD31 is constitutively expressed on the surface of circulating monocytes. Others have found that many circulating progenitor cells express monocyte surface markers and function predominantly through secretion of angiogenic factors

(124). In fact, it has been shown that the majority of cells localized around newly-formed blood vessels are positive for the macrophage marker F4/80. Interestingly, F4/80 + and CD31+ cells do not exhibit markers for classic EPCs, suggesting that that bone marrow-derived angiogenic monocytes are actually playing a large role in supporting angiogenesis that was originally thought to be reserved for progenitor cells (51). Circulating blood macrophages expressing CD31 have also been found to contribute to the formation of new blood vessels. In a parabiosis mouse model, CD31+ macrophages tagged with GFP were present in the walls of new vessels in wounds, gel foam blocks and tumors of the wild-type mouse, demonstrating that monocytes expressing both CD31 and macrophage surface markers could be recruited to areas of tissue injury to assist in repair and new blood vessel formation (77).

In young healthy men, CD31+/CD34- cells positive for the monocyte marker CD14 exhibit significantly higher levels of intracellular ROS after consumption of a high fat meal, which is completely prevented with prior endurance exercise (64). This indicates that intracellular redox balance in CD31+ monocytes is affected by environmental stimuli, and emphasizes the importance of studying the health of CAC when considering these cells for autologous therapies. Indeed, reports of contribution of CD31+ cells in the development of atherosclerosis in areas of disturbed flow in ApoE-deficient mice indicate that these cells can also have inflammatory properties (42). A recent study found that CD31+ cells from human patients with coronary artery disease (CAD) exhibited impaired angiogenic potential and expressed lower levels of pro-angiogenic genes compared to healthy counterparts. There were significant correlations between the number of CD31+ cells and severity of coronary artery disease (76).

Interestingly, this study performed flow cytometry analyses to characterize the cells and found that the selected CD31+ cells of CVD patients were composed mainly of monocytes/macrophages (CD14 and CD11b), whereas the selected CD31+ cells of healthy individuals were composed of mostly T-cells (CD3) (76). Both cell types have been found to secrete factors that promote angiogenesis, however, monocytes and macrophages have also been associated with inflammation. This study also found an upregulation of pro-inflammatory genes and down regulation of angiogenic genes in the CD31+ CAC of CAD patients compared to healthy controls, suggesting that the cells from this patient population may be involved in the inflammatory process of atherosclerotic plaque development (76). These authors conclude that CD31+ cells from CAD patients may serve as a source of inflammatory factors contributing to the etiology of CAD. For this reason, caution must be used when using autologous CD31+ cells for therapeutic purposes to treat patients with established CVD.

To our knowledge, there have been no clinical trials to date that utilized purified CD31+ cells for autologous therapy in humans. However, there are a number of advantages to using CD31+ cells as opposed to EPCs or other circulating stem cells for therapeutic purposes. Among these advantages include a greater abundance of CD31+ cells present in circulation, and the ability to use these cells after isolation from PBMCs without the use of *in vitro* culture (75). More than 90% of hematopoietic stem cells, multipotent progenitor cells, lymphoid progenitors and monocyte progenitor cells express CD31 (78). In human bone marrow derived cells, nearly 100% of CD34+/CD133+ cells were positive for CD31 and 89% of CD34+/CD133- cells were positive for CD31 (78). In addition, CD31+ have greater adhesion capacity to extracellular matrices such as laminin, collagen and fibronectin compared to CD31- cells giving them a greater chance of survival and engraftment when used for therapeutic purposes (78).

Discordant Findings in CAC Therapies

As the use of CACs for therapy for ischemic CVD is a relatively new concept, it is important that the major differences in CAC treatments are explored to best optimize the therapeutic use of CACs for both primary and secondary CVD therapy. To this day, the agreement on the best type of CAC for autologous therapy has been disputed (Bone marrow, PBMC, preselected, culture, etc.). Many trials use bone marrow aspirations as an attempt to isolate more immature cells with greater plasticity. However, the results of these trials are not very successful (37, 120, 121), suggesting that more differentiated or mature cells may have a greater functional capacity. Along the same lines, total MNCs are still being used for some trials. The rationale behind the use of total MNCs as opposed to a single selected cell type, involves the physiological relevance as *in vivo* conditions involve a complex cell-cell communication with other circulating immune and hematopoietic cells. While this may be true, it does appear based on the studies listed above, that the CD34+ and CD31+ populations are of critical importance and have overall better outcomes compared to MNCs (72, 120, 125).

Similarly, the protocol for isolating or identifying these cells as well as the model used to test their function can also affect the clinical relevance. For example, it has been suggested that OECs are products of angiogenic culture conditions and, therefore, may not exist in the systemic circulation (29). How these cells would then behave if re-introduced to the body is not yet established. Additionally, there are various combinations of cell markers that are currently being used in clinical settings. The greater number of cell markers required means these cells become more rare, limiting what they can be used for. The type of "injury" induced in animal models (hind limb ischemia, experimental MI, denuded mouse, etc) could affect the outcomes of the study in the case that the specific stimuli preferentially recruit certain subsets of CACs. Along the same line, since many of the CD34+ subpopulations of CACs are very rare *in vivo*, some investigators use mobilizing agents to recruit more cells. The effects of these mobilizing agents on the CACs function have not yet been well explored.

The dose or potency of the CAC treatment has also been explored with lower cell doses of more purified cell populations showing the most success (95). Additionally, the mode of delivery (intracoronary injections, systemic, pericutaneous, etc.) may also play a role in the ability of the CAC to reach the site of injury and in long-term retention. Finally, the timing of treatment based on disease severity or time following an acute event is also critical and it is still not optimized.

The best outcome measure to test whether the CAC therapy is successful is also not yet established. To date, the majority of clinical trials using CACs to treat MIs use left ventricular ejection fraction as a major outcome measure. However, a more recent study (115) suggests that for detection of improvements in CAC therapy after a MI, a better indicator is to measure infarct size. Thus, it is possible that as we learn more about CACs, we will also find better means of establishing CAC function *in vivo* and functional outcomes of the heart or other ischemic tisses as a result of CAC therapy.

Finally and potentially one of the largest contributors to the discordant findings in the therapeutic use of CACs to treat CVD has to do with the autologous nature of the cells. In many cases, CACs are being harvested from patient populations. As mentioned earlier, there is a negative relationship between the function of CACs and the severity of CVD. Thus, CACs from patients who recently suffered an acute event likely have lower functioning CACs compared to healthy adults. Since these lower functioning cells are being re-introduced this could contribute to the inconsistent findings with these treatments.

Role of Oxidative Stress, Nitric Oxide and Inflammation on CAC Function

As mentioned above, one of the critical issues with the use of autologous CAC therapy has to do with the fact that the CACs isolated from patient populations, those who might require stem cell therapy are low-functioning compared to healthy individuals. The cells from most CVD patients are exposed to chronically high levels of inflammation and oxidative stress and are often from individuals older in age. Indeed, lower functioning CACs have been identified in cases of atherosclerosis/CAD, obesity, type II diabetes, etc. with the function of CACs declining with the addition of more risk factors (35) (Figure 2.3). For example, in the metabolic syndrome, the combination of obesity, dyslipidemia, hypertension and hyperglycemia all contribute to the decreases in CAC function and improvements in even just one factor has been shown to elicit some improvements (14, 35). This has also been evident in cases such as smoking cessation and exercise training, two interventions that are associated with decreasing whole body oxidative stress and inflammation (147).

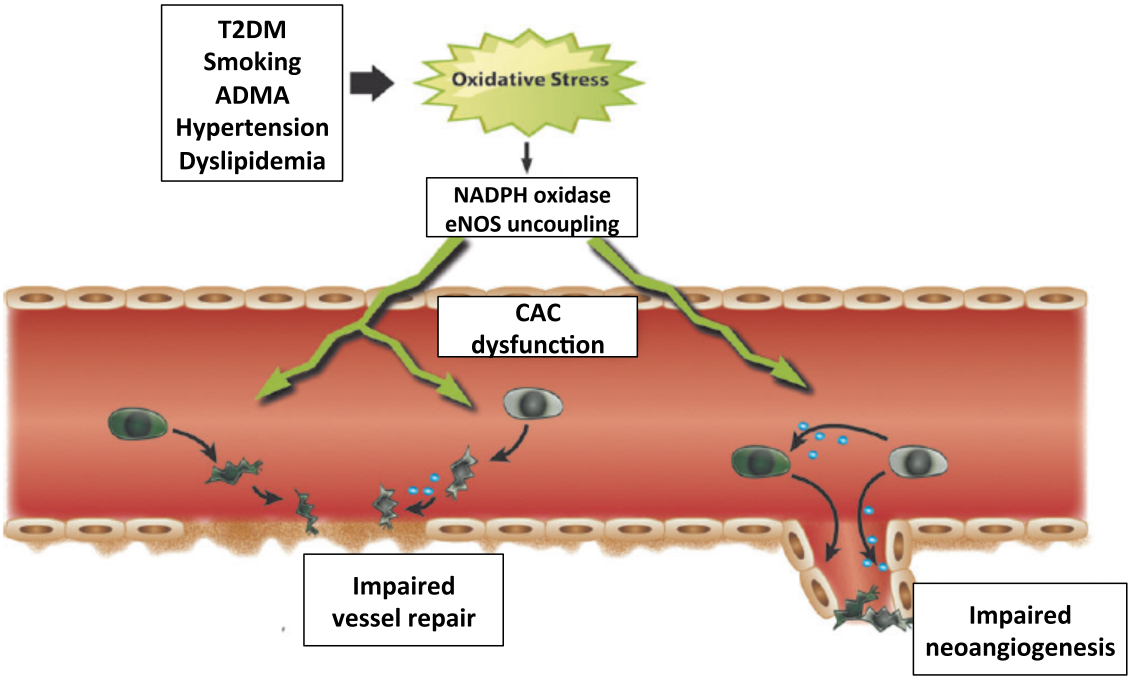

T2DM
Smoking
ADMA
Hypertension
Dyslipidemia
Oxidative Stress
NADPH oxidase
eNOS uncoupling
CAC
dysfunction
Impaired
vessel repair
Impaired
neoangiogenesis

Figure 2.3 A number of environmental and pathophysiological factors generate oxidative stress, which affects CAC function and consequently impair endothelial repair. Oxidative stress results in greater levels of NAHPH oxidase and eNOS uncoupling leading to CAC dysfunction. Dysfunctional CACs cannot adequately contribute to vessel repair or neoangiogenesis. Figure adapted from Fleissner and Thum (2011)(35).

The common factor in most of these chronic conditions and likely the greatest contributor to CAC dysfunction is decreased NO bioavailability. A key study that addressed the critical importance of eNOS and NO in CAC number and function was performed by Aicher et al. (2003) who found that revascularization after hindlimb ischemia was impaired in eNOS knockout mice. However, when CACs from wild type animals were introduced into the animal this affect was attenuated (1). This study emphasized the critical importance of eNOS-derived NO at the site of neovascualrization in order for CACs to function properly. Indeed, eNOS- derived NO is a major regulator of both extracellular factors mediating mobilization of CACs from the bone marrow as well as the intracellular function of CACs. In a healthy young adult, extracellular physiological NO activates MMP9 within the bone marrow, which, after subsequent c-kit ligand splicing, mobilized CACs from the bone marrow into the circulation (35). Once in the circulation, CACs travel to an area of damage based on chemokine attraction. The ability of the CAC to perform this task involves physiological intracellular NO. eNOS activation within the CAC results in an increase in VEGF expression, which contributes to the proliferative and angiogenic properties of the cell. eNOS-derived NO also contributes to cytoskeletal changes and increases in vascular-stimulated phosphoprotein (VASP), which improve the migratory responses of CACs to the angiogenic stimuli coming from the site of injury. eNOS-mediated NO can also affect transcription factors within the cell nucleus and, thus, transcription of angiogenic genes, further enhancing the vasculo-protective and angiogenic capacity of CACs (35).

The endogenous eNOS inhibitor, asymmetric dimethylarginine (ADMA) is elevated in disease conditions such as coronary artery disease and atherosclerosis. Elevated extracellular levels of this factor can be taken up by CACs and lead to decreased NO bioavailability (35). One mechanism through which this has been found to occur involved the up regulation of microRNA-21 in CACs in response to high circulating ADMA concentrations. miR-21 targets superoxide dismutase 2 resulting in a repression of antioxidant capacity and decreased protection against oxidative stress. miR-21 also targets sprouty-2 which results in an increase in intracellular oxidative stress through an MAPK/ERK dependent mechanism. This combination between increased intracellular oxidative stress and decreased protection from oxidative stress contributes to the ROS-dependent decrease in intracellular NO bioavailability (35). Together this creates a dysfunctional CAC with impaired ability to repair injured endothelium (Figure 2.4).

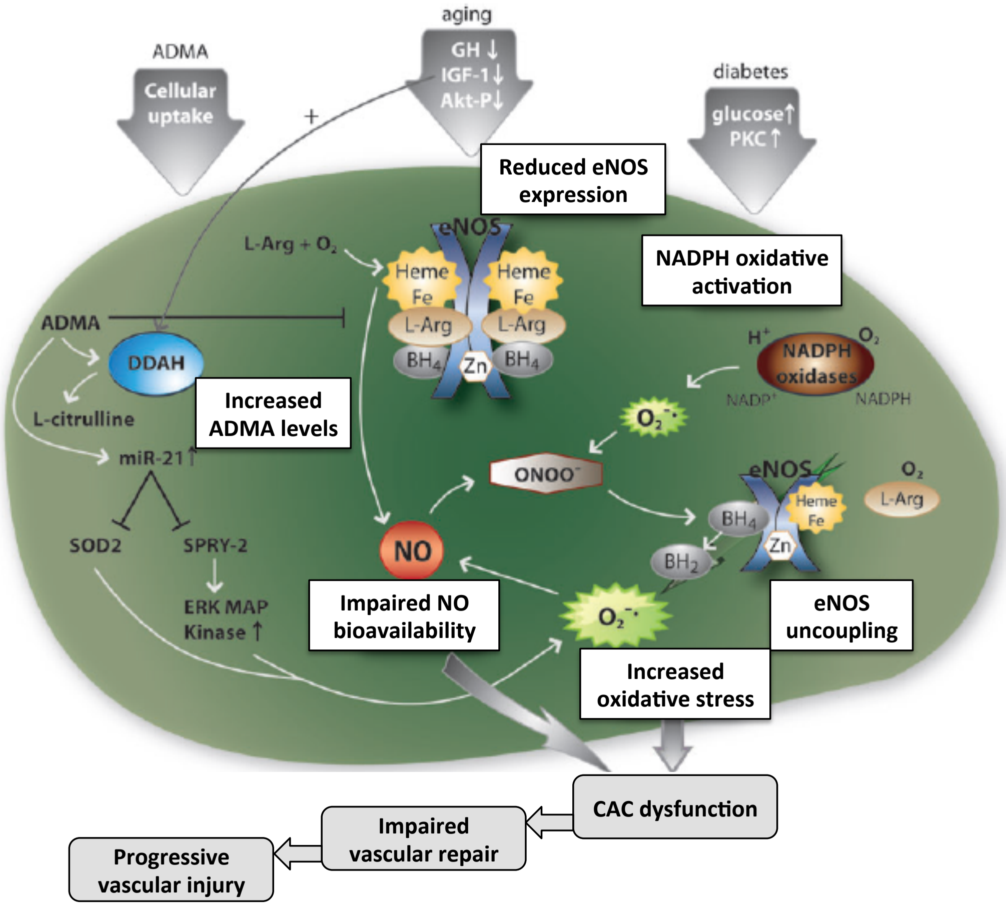

aging
GH ↓
IGF-1 ↓
Akt-P ↓
ADMA
Cellular uptake
diabetes
glucose ↑
PKC ↑
+
Reduced eNOS expression
eNOS
L-Arg + O₂
Heme Fe
L-Arg
BH₄
Zn
NADPH oxidative activation
ADMA
DDAH
L-citrulline
Increased ADMA levels
miR-21 ↑
SOD2
SPRY-2
ERK MAP Kinase ↑
H⁺
O₂
NADPH oxidases
NADP⁺
NADPH
O₂•⁻
ONOO⁻
NO
eNOS
Heme Fe
BH₄
BH₂
Zn
O₂
L-Arg
Impaired NO bioavailability
eNOS uncoupling
O₂•⁻
Increased oxidative stress
CAC dysfunction
Impaired vascular repair
Progressive vascular injury

Figure 2.4 A number of factors such as age, diabetes and ADMA can affect intracellular signaling of CAC and lead to CAC dysfunction, which consequently impairs vascular repair and leads to progressive vascular injury. Figure adapted from Fleissner and Thum (2011) (35).

Aging is associated with an increase in visceral adiposity, increased oxidative stress and inflammation, and decreased production of growth factors (IGF-1, GH, VEGF). Growth factors affect the health of CACs by activating eNOS and up regulating NO bioavailability (35). VEGF is also involved in the mobilization of CACs from the bone marrow and migration of CACs to ischemic tissue. In older adults, where the production of these growth factors is compromised, there is a decrease in intracellular eNOS activation resulting in decreased NO bioavailability and a dysfunctional CAC, impaired repair capacity and as a result, further progression of the disease (Figure 2.4).

Inflammation associated with obesity also can have detrimental effects on CAC function. In an atherogenic environment CACs can become activated and take on more of a monocytic fate where they infiltrate into the subendothelial space and turn into foam cells, further secreting angiogenic and inflammatory factors (21, 56, 100, 105). Initially this is an attempt to stabilize plaque development, but over time these foam cells can contribute to an unstable plaque and plaque rupture. The ability of CACs to take on a foam cell phenotype is affected by the extracellular environment and an increase in pro-inflammatory factors and lipids often found in obese populations (21, 100, 169). Additionally, oxidized LDL has been found to decrease eNOS activity and NO bioavailability, while lipid apheresis is reported to rescue eNOS activity and enhance CAC function (35).

Hyperglycemia and type II diabetes are other conditions that lend to an extracellular environment with elevated oxidative stress and inflammation (Figure 2.4). High plasma glucose affects CACs by increasing levels of NADPH oxidase leading to eNOS uncoupling, thereby decreasing NO bioavailability and further increasing intracellular ROS (139). *In vitro* assays have found that CACs cultured in a high glucose environment exhibited decreased adhesion, lower proliferative capacity and impaired *in vitro* tube formation (67).

The notion that a variety of diseases can modify CAC function has become increasingly recognized. As such, and due to the autologous nature of CAC therapies, researchers have been looking for ways to enhance the function of CACs from patient populations before using them for transplants. Mackie et al., found that genetically modifying CD34+ CACs to overexpress sonic hedgehog (shh) better preserved cardiac function when these cells were injected into mice after experimental acute MI (102). Other labs have used adenoviral infection to upregulate antioxidant or angiogenic pathways. *Ex vivo* transduction of CACs with akt/HO-1, key factors involved in cell survival and angiogenesis, resulted in decreased CAC senescence and increased protection from pro-oxidative factors (13). Additionally, adenoviral infection with VEGF has been found to improve the ability of CACs to respond to hindlimb ischemia via increased neovascularization and increased paracrine release of VEGF (58). These studies suggest that the future of CAC therapy might lie in the ability to enhance the function of cells from diseased individuals, perhaps using a combination of factors that are enhanced or downregulated in optimally functioning populations.

Exercise and CAC Function

Recently, there has been an emphasis on improving the function of CACs to be used for autologous cell therapies to treat lower functioning CACs from individuals with known pathologies and/or enhance their reparative properties. However, it is important to consider potentially negative outcomes associated with genetic or pharmacological treatments if the proper balance is not found. There have been a number of studies that explore the effects of both acute and chronic exercise on different populations of CACs in healthy and diseased populations (82, 156). The vast majority of these findings provide support for the beneficial effects of exercise to enhance CAC function. Our lab has identified young endurance exercise trained individuals as an ideal population to guide mechanistic intervention therapies to enhance CAC function. Indeed, we have found that aerobic exercise is an ideal treatment for both enhancing CAC function as a preventive measure and as a model to allow assessment of the molecular mechanisms associated with optimal CAC function. Thus, identification of the mechanisms underlying the improved extracellular and intracellular environment in exercise-trained individuals provide potential targets to improve the function of CACs and could serve as a promising therapy for CVD.

Acute Exercise

Acute aerobic exercise is a known stimulus for increasing circulating concentrations of CACs. Mobius-Winkler et al. (2009) found that cycling continuously for 4 hours at 70% of an individual's ventilatory threshold caused a time-dependent release of CD34+ and CD34+/VEGR2+ CACs. They noted that number of both CAC populations was highest 3.5-4 hrs into the cycling bout (109) and slowly declined over a period of a few hours upon completion of exercise. It was also discovered that the rise in CACs was preceded by an early rise in VEGF and later, IL-6 which are believed to serve as signals to mobilize CACs from bone marrow and into the circulation (109). Another study found that an acute exercise bout substantially shorter than that in the Mobius-Winkler paper (~20 min vs 4 hrs) significantly increased circulating CD34+ CAC number in both young and older men (138).

Importantly, differences in CAC number and function have also been observed in younger adults as a function of chronic aerobic exercise habits. Jenkins et al. (2009) observed a differential response to an acute submaximal exercise bout in younger inactive vs. active adults (66). No differences in CFU numbers were observed at baseline, however, mRNA levels of the pro-oxidative enzyme gp91phox were higher and eNOS mRNA was lower in the CACs of inactive relative to active individuals. After the acute exercise CFU number increased in the active group only and corresponded with reduced mRNA expression of pro-oxidative enzymes. Conversely, acute exercise increased inducible NO production in inactive individuals (66). Another study by Jenkins et al (2011) found that increases in intracellular ROS in CD31+ CACs of younger adults caused by consumption of a high fat meal were completely prevented when an acute bout of exercise was performed on the prior day (64).

Acute exercise can also have a beneficial effect for diseased populations. Chronic heart failure patients with impaired endothelial and CAC function exhibited improvements in CAC migratory function with just one acute bout of exercise (146). In the same study, CD34+ and CD34+/VEGFR2+ CAC number, however, remained unchanged. Beneficial effects have also been observed in PAD patients after one acute bout of exhaustive exercise. Although numbers of CD34+/VEGFR2+ CACs were similar at baseline between healthy controls and PAD patients, CD34+/CD133+ CACs were lower in PAD subjects and acute exercise significantly increased CD34+/CD133+ CAC number (135). As one acute bout of exercise has the ability to improve CAC number and function, we can speculate that regular participation in aerobic exercise would provide a overall protective effect on CACs.

Exercise Training

It is well known that regular aerobic exercise training is associated with improvements in circulating lipid profiles, reductions in oxidative stress, decreased inflammatory response, and up regulation of endogenous antioxidant capacity, all of which contribute to an increase in NO bioavailability. Regular aerobic exercise is also associated with increases in CAC number in both healthy and diseased populations, regardless of age, and this is typically correlated with improvements in endothelial function (166, 170). In one study, older and younger men participating in 30 min of aerobic exercise 3 times/week for 3 months showed significant elevations in CD34+/KDR+ CACs and this was associated with decreases in brachial-ankle pulse wave velocity (baPWV) (170). In fact, after the 3 month training period, CAC number, migratory capacity and proliferative activity as well as baPVW in older adults was similar to those of younger adults, suggesting that regular aerobic exercise can attenuate some of the age-related decline in arterial elasticity via increased CAC number (170).

The effects of exercise on CAC number and function are at least partially mediated by shear stress. Indirectly, shear stress acting on the endothelial wall results in increases in VEGF and NO, which are known to mobilize CACs from the bone marrow (35). Once in circulation, and/or localized to an area off damage, shear stress acts directly on the CACs through various signaling pathways (117, 168) to enhance CAC function. In fact, one study exposed lower functioning CACs from older adults to shear stress and found improvements in cell migration, adhesion, and re-endothelialization capacity of these CACs (168). There is also evidence that exposure of CACs to shear stress upregulates integrins β_1 and β_3, which results in increased adhesion and differentiation toward a more endothelial phenotype (20).

Another study assessed at the effects of 3 months of regular aerobic exercise on CFU CACs in middle-aged and older men and found that although CAC migration was initially reduced in older compared with young and middle aged men, the exercise intervention increased both CFU number and migratory capacity in both middle-aged and older adults (50). Interestingly, another study examining the effect of ageing and exercise on CACs found that irrespective of age, aerobic exercise training (8 weeks, 3x/wk for 30 min betwwen 65-85% heart rate reserve) did not affect baseline or acute exercise-induced numbers of CD34+ CACs (138). Similarly, Witkowski et al. (2010) found no difference in baseline numbers of CD34+ or CD34+/VEGR2+ CACs of CFU colonies in older inactive individuals compared to masters athletes, but did see a substantial reduction in CD34+ cell number after just 10 days of exercise cessation in the older adults. These findings were closely related to a physical activity dependent changed in reactive hyperaemic forearm blood flow measurements (166). As advanced age is an independent risk factor for developing CVD, it is promising to consider the preventive effects of aerobic exercise in older populations to protect them from potential future cardiac events.

Importantly, differences in CAC number and function have also been observed in younger adults as a function of chronic aerobic exercise habits (10). Intracellular ROS and NO levels were found to be significantly higher in the CD34+ CACs from inactive men compared to endurance-trained men along with greater iNOS and lower eNOS mRNA expression, suggesting a link between the physical inactivity and nitro-oxidative stress (63). Importantly, these differences were eliminated after one acute bout of exercise (63). Another study assessed the effects of reduced physical activity on CD34+ CACs and CFU CACs in younger adults. They found that 10 days of reduced physical activity resulted in lower CFU CAC numbers and reduced intracellular NO levels, but had no effect on CD34+ CAC number or intracellular NO (40), suggesting that the effects of exercise on CAC number and function might be cell-type specific. A recent study in our lab found significant differences in paracrine activity of CD34+ and CD34-/CD31+ CACs as a function of habitual activity with lower HUVEC tube formation observed when CM from inactive individuals was used compared to CM from active and high endurance-trained individuals (87). Additionally, it appears that the differences in tube formation were at least partially due to a greater secretion of S100A8 and S100A9 proteins from CD34-/CD31+ CACs of inactive individuals. Interestingly, in this study, there were no observed differences in intracellular ROS or NO as a function of chronic exercise habits (87).

Although as a whole, exercise training does appear to be beneficial for CAC function, it is important to note that there have been some studies that did not observe differences in CAC number as a result of training. Rakobowchuk et al. (2012) found that CD34+ CAC number was higher after 6 weeks of heavy interval exercise training, although high variability among subjects made this increase actually not statistically significant (122). The subjects in this study were younger healthy individuals, suggesting that in populations already exhibiting high CV function, the effects of exercise on CAC mobilization can be highly variable.

It is important to study CAC number and function in younger healthy individuals of varying levels of physical fitness to determine whether differences can be detected before classic CV risk factors are observed. However, as individuals with various forms of CVD are the target group theoretically receiving CAC therapy, it is vital that we identify what properties of CACs are limiting the potential of these cells to be successfully utilized for autologous treatments and whether mechanisms associated with exercise can help improve this. Indeed, exercise training can also have beneficial effects of CAC number and function in a number of CVD settings (33, 91, 131, 136, 146, 148). For instance, CACs from individuals with metabolic syndrome have decreased functional capacity when tested in a nude mouse denuded artery model. After 8 weeks of exercise training, CACs from these same individuals significantly improved re-endothelialization and decreased size of denuded area (136). Exercise training has also been beneficial for heart failure patients who exhibit CAC and endothelial dysfunction compared to healthy controls. Van Craenenbroeck et al. (2010) enrolled chronic heart failure patients in 6 months of exercise training and found that CD34+/VEGFR2+ CAC number and migratory capacity were substantially improved along with improvements in peripheral endothelial function (148). Six months of supervised walking can also improve CAC number and endothelial function, accompanied by decreases in the NO-inhibitors ADMA in patients with peripheral artery disease (PAD)(131). Interestingly, these benefits were not observed in a group receiving just medical treatment for PAD. It is important to note that structured exercise programs are not required in order for beneficial changes in CAC number and function to take place. Activities of daily living have been shown to be positively correlated with CD34+/VEGFR2+ CAC number, with total activity being positively associated with colony forming capacity of CD34+ CACs in coronary artery disease patients after suffering from a MI (83).

The promising effects of exercise training have begun to enter into the clinical realm as Domanchuk et al. (2013) has initiated a PROgenitor cell release Plus Exercise (PROPEL) trial to improve functional performance in patients with PAD. This study will examine whether granulocyte-marophage colony stimulating factor (GM-CSF) treatment combined with supervised treadmill walking or each therapy alone will improve 6-minute walk distance, brachial artery flow mediated dilation (FMD) and CD34+ cell number after 12 weeks (24). As there are a number of benefits to exercise in addition to improved CAC function, it is likely that more clinical trials such as this one will be performed in the future to assess them as viable options for treating CVD.

Paracrine Functions

As described briefly above, there are some cases of both CD34+ and CD31+ CACs engrafting into the endothelium (4). However, studies using GFP tagged CACs for bone marrow transplantation (175) or in a parabiosis model have found that very few, if any, transplanted CACs actually incorporate into the existing vasculature. One study transplanted bone marrow expressing GFP in mice and 6 weeks after transplantation the mice underwent unilateral femoral artery occlusion. Investigators found that despite improvements in hindlimb collateral artery growth, there was no GFP signal within endothelial or smooth muscle cells (173). They did, however, find significantly greater accumulations of GFP-tagged cells alongside collateral arteries in the ischemic limb compared to the non-occluded side. These findings support the notion that incorporation of bone marrow-derived CACs into the vasculature is not required for angiogenic effects, rather they are likely functioning as supporting cells to aid in angiogenesis (175).

It is now generally accepted that CACs function predominantly through paracrine mechanisms. One of the first major reports of CAC paracrine activity assessed secreted factors from CACs cultured in normal and hypoxic conditions. They found a number of angiogenic cytokines in the media including VEGF, PIGF, bFGF and MCP-1 after 72 hours and the CM from these CACs promoted endothelial cell migration and proliferation *in vitro* (80, 81). Additionally, *in vivo* experiments using a mouse hindlimb ischemia model found enhanced recovery, greater collateral artery growth, improved limb function and lower muscle atrophy when CM was used compared to control media (80). This and other studies performed by this lab also demonstrated that CACs assist in collateral remodeling after hindlimb ischemia in mice through paracrine mechanisms (80, 81). Other studies have found that CM derived from CACs helped reduce myocardial inflammation associated with autoimmune myocarditis (49) and myocardial infarction (54). *In vitro* studies have found that co-culture of CD34+ CACs with HUVECs, so that they shared the same environment but were kept from directly interacting, resulted in increased sprout lengths of HUVEC spheroids (129) and identification of a number of secreted factors in the shared CM.

Several angiogenic factors have been identified in the secretome of various types of CACs. To date, the vast majority of research has been focused on secreted proteins (80, 81, 145, 168), namely angiogenic factors or growth factors with known roles in angiogenesis. However, more recently there has been a body of literature directed at the identification of microRNAs secreted by CACs. Recently, microRNA-126 and microRNA-130a have been identified in the cell secretome of CD34+ CACs, primarily packaged within exosomes and that these are largely mediating the observed proangiogenic effects of these cells (125). Mocharla et al. (2013) found that CD34+ cells and CM derived from CD34+ CACs exerted superior proangiogenic actions as assessed by tube formation compared to CM from CD34- CACs. They also discovered that microRNA-126 was secreted from CD34+ CACs in exosomes and that inhibiting the release of microRNA-126 by transfection of CACs with anti-miR-126 impaired these proangiogenic actions. Angiogenic actions were also impaired due to reduced secretion of microRNA-126 from CD34+ CACs from individuals with type II diabetes, but treatment with a microRNA-126 mimetic rescued these actions (110). Although it is also believed that CD31+ CACs function predominantly through paracrine functions as well, secretion of exosomes and accompanying microRNAs from this subset of CACs has not yet been well explored. These studies emphasize the importance of exploring secreted factors in addition to just cytokines that may be mediating the proangiogenic effects of CACs.

The mechanisms behind the paracrine activities of CACs have not yet been fully explained. It has been found that overexpression of integrin β5 enhances the reparative paracrine functions of CACs through phosphorylation of integrin αvβ5 and subsequent activation of Src Kinase and signal transducer and activator of transcription (STAT)3. CM from these CACs successfully enhanced human endothelial cell sprouting using a spheroid assay compared to CM from vector-treated CACs (90). They also found elevated levels of angiogenic cytokines CCL2 and CXCL8 in the CM of the integrin β5 enhanced CACs.

As our lab has previously found, both acute and chronic physical activity and diet can affect the intracellular redox balance within both CD34+ and CD31+ CACs (63, 64, 66). However, we have since found significant differences in angiogenic tube formation in both CD34+ and CD34-/CD31+ CACs as a function of chronic physical activity in younger men and women despite no observed differences in intracellular ROS and NO. As such, it appears that at least in the case of younger individuals, paracrine function is not mediated by intracellular redox balance (88). However, other studies have found that antioxidant supplementation affects cytokine production by monocytes after an acute bout of exercise, suggesting that if redox balance is involved, the mechanisms may be cell-specific or require stimulation of some cells (i.e.- exercise) to have a significant effect (141). Other mechanisms affecting the paracrine function of CACs, to our knowledge, have not yet been explored.

Although some focus has been placed on identifying mechanisms of paracrine release as a consequence of intracellular health, it is also likely that paracrine factors are differentially released or expressed depending on the microenvironment that the CACs encounter (38). The released factors can then affect the surrounding tissues and/or the CACs themselves as a result. Indeed, environmental stimuli such as ischemia can alter Akt-mediated release of soluble factors from CACs, which then act in an autocrine manner to influence further CAC actions (i.e. proliferation) or a paracrine manner, acting on surrounding endothelial cells, smooth muscle cells or cardiomyocytes to induce repair or regeneration (38).

As mentioned above, the health of the cell donor is one of many factors that may be contributing to some of the discordant findings among CAC therapies. To date, the majority of studies investigating paracrine actions of CACs have focused on growth factors that are believed to enhance angiogenesis. As it is generally accepted that CACs from older or diseased individuals function poorly, we can assume that this means there is a good chance that these CACs are potentially secreting pro-inflammatory or anti-angiogenic factors that may disrupt “normal” angiogenic behaviors. As mentioned previously, we have found that there are significant differences in the secretion of inflammatory proteins S100A8 and S100A9 from CD34-/CD31+ CAC as a function of habitual physical activity levels in younger healthy adults. CM from CD34-/CD31+ CACs of inactive individuals contained twice as much S100A8 and S100A9 as CM from their endurance-trained counterparts. Additionally, tube formation was reduced when concentrations of S100A8 and S100A9 similar to those found in the CM of inactive individuals' CACs were used in the assay (88). These findings suggest that these proteins, and possibly others, are at least partially explaining the differences in CAC function found as a result of lifestyle.

S100A8/S100A9

Structure and Function

S100A8 (aka MRP8 or calmodulin A) and S100A9 (aka MRP14 or calmodulin B) are small molecular weight proteins (11 KDa and 14KDa, respectively) that belong to the S100 family of calcium-modulated proteins. They are abundantly expressed in myeloid cells and their expression may be related to a certain stage of myeloid differentiation, as some studies indicate that expression is present in neutrophils and monocytes but is no longer evident in mature macrophages (74), while others have found that CD68+ macrophages in rheumatoid arthritis patients expressed antigens for S100A8/A9 (137). Meanwhile, others have found that expression patterns of both S100A8 and S100A9 in a number of cell types are dependent on the type and severity of the inflammatory condition (74). Expression of S100A8 and S100A9 has also been found in some epithelial and vascular cells (74, 153). However, surface expression of S100A9/A9 is not required for secretion of these proteins (74). Importantly, S100A8 and S100A9 are both expressed and secreted by CACs (31, 88, 145).

S100A8 and S100A9 are released by a novel secretory pathway that is distinct from the classic endoplasmic reticulum/Golgi pathway (123, 153). Secretion is dependent on an intact microtubule network and involves the activation of protein kinase C (74, 123). However, there is also evidence of passive release of S100A8/A9 from necrotic cells after tissue damage (153).

S100A8 and S100A9 can function independently as homodimers, bound together as a S100A8/S100A9 heterodimer (aka calprotectin), as well as in heterotetramer forms (74, 153). Self-assembly of these proteins into different structures is largely influenced by the Ca^{2+} and Zn^{2+}- binding properties. Indeed, elevated Ca^{2+} levels present in mononuclear cells in inflammatory environments trigger the cytosolic monomers of S100A8 and S100A9 to translocate to the cell membrane where they form the more stable heterodimer complex (27). Furthermore, the structure of these proteins has been found to dictate their function, leading to a wide functional diversity of S100A8 and S100A9 (153) with the heterodimeric complex being the predominantly occurring, most stable and most physiologically relevant form (18, 48, 153). Eue et al., demonstrated that S100A8 and S100A9 can specifically bind to human endothelial cells with enhanced binding capacity of S100A9 over S100A8 and the heterodimer complex having the greatest capacity to bind to endothelial cells (27). At least one study has also found that S100A8 can regulate S100A9 activity through formation of the S100A8/A9 heterodimer (116). It should be noted that multiple forms of these proteins can exist in parallel and currently, there is no method for detecting the fractions of homodimers, heterodimers or functional effects of each of these if all are present simultaneously (153).

These proteins can perform a wide variety of intracellular and extracellular functions and are involved in both normal physiological signaling and processes and a number of disease pathologies including inflammatory conditions, autoimmune diseases, cancer and atherosclerosis (74, 153). High levels of S100A8/A9 in the circulation have been found in inflammatory disorders such as cyctic fibrosis, rheumatoid arthritis, and crohn's disease (137). More recently, high systemic S100A8/A9 levels have also been linked to a number of different CVDs (5, 17, 18, 43, 130, 153) and also to several CV risk factors such as smoking, hyperlipidemia, hyperglycemia, and obesity (130) (Figure 2.5)

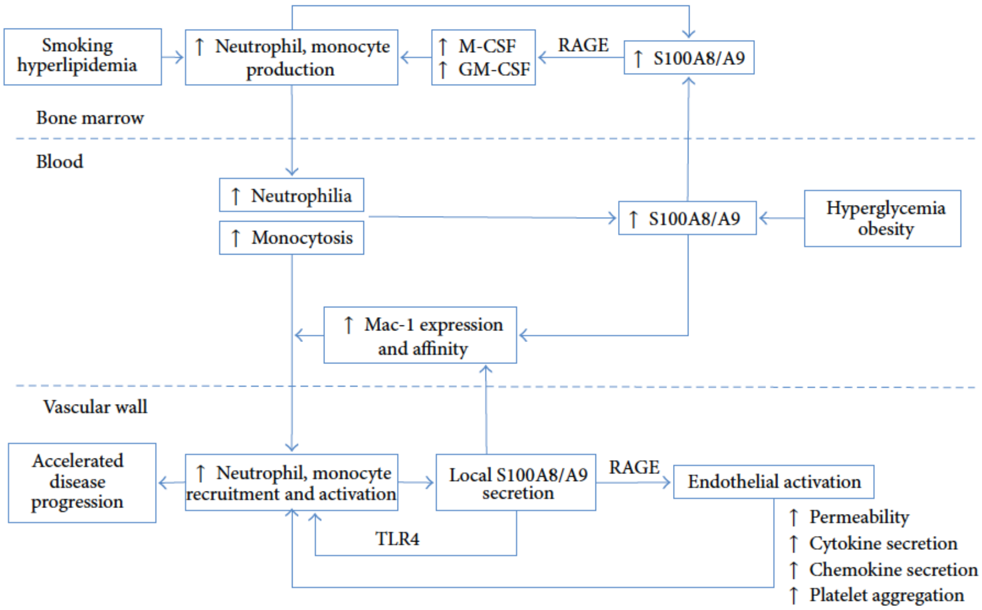

Smoking hyperlipidemia
↑ Neutrophil, monocyte production
↑ M-CSF
↑ GM-CSF
RAGE
↑ S100A8/A9
Bone marrow
Blood
↑ Neutrophilia
↑ Monocytosis
↑ S100A8/A9
Hyperglycemia obesity
↑ Mac-1 expression and affinity
Vascular wall
Accelerated disease progression
↑ Neutrophil, monocyte recruitment and activation
Local S100A8/A9 secretion
RAGE
Endothelial activation
TLR4
↑ Permeability
↑ Cytokine secretion
↑ Chemokine secretion
↑ Platelet aggregation

Figure 2.5. Overview of the interplay between S100A8 and S100A9, traditional CV risk factors, circulating phagocytes, and atherogenesis. Figure retrieved from Schiopu et al. (2013) (130).

S100A8 and S100A9 function in a cytokine- or chemokine-like manner through binding to one of their many receptors, however the precise functions and molecular signaling mechanisms are not completely understood (74, 118). Receptors for S100A8 and S100A9 include the receptor for advanced glycation end products (RAGE), Toll-like receptor 4 (TLR4), and CD36 (11, 26, 153, 155, 162). There are some reports that S100A8 preferentially binds to TLR4 while S100A9 preferentially binds to RAGE (153, 162), both of which are present on endothelial cells. Both homodimers and the heterodimer complex have the ability to interact with either receptor, which are often present on the same cell line, but it is believed that only one receptor is likely used at a given time (153). Downstream of these receptors, a signaling cascade is initiated which results in the phosphorylation of ERK1/2 and p38 leading to activation of the transcription factor nuclear factor-κB (25, 137, 153, 162) (Figure 2.6). Upon binding to their receptors, S100A8/A9 can perform a number of functions including stimulation of neutrophil adhesion via β_2 integrins (74), and promotion of leukocyte trafficking and leukocyte-endothelial cell interaction (154)leading to activation of the microvascular endothelium, loss of endothelial barrier function and upregulation of thrombogenic factors (5, 74, 153, 159, 162).

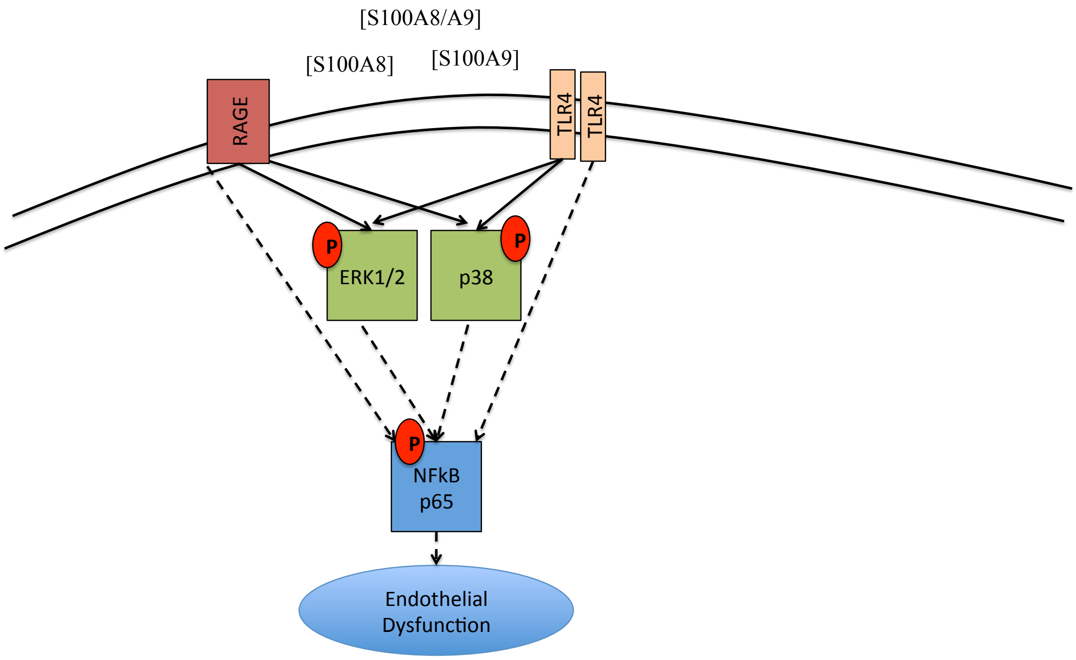


Figure 2.6. Proposed pathway through which S100A8/S100A9 act on endothelial cells. S100A8 and S100A9 bind to two major receptors present on endothelial cells, RAGE and TLR4, which triggers downstream signaling pathways that result in endothelial dysfunction. Figure adapted from Wang et al. (2014) (162)

Indeed, the role of S100A8/A9 in CVD has recently become clearer. Elevated circulating levels of S100A8/A9 are considered independent risk factors for a number of CVDs (71) with plasma levels predicting future CV events in middle-aged healthy individuals (17, 18, 43). One study found that serum levels of S100A8/A9 were significantly higher in patients with an acute MI compared to patients with unstable angina and that the levels of these proteins in the MI patients positively correlated with peak white blood cell and neutrophil counts, and peak levels of creatine kinase and C-reactive protein (71). These proteins also play a functional role in the pathogenesis of inflammatory CV disorders with an activated endothelium being a major stimulator for surrounding monocytes to secrete S100A8/A9 and promote adhesion and transendothelial migration of inflammatory cells (27). For example, TLR4 activation by S100A9 promotes an inflammatory response through activation of NF-κB, which induces expression and secretion of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-10, and TNF-α (137). Similarly, HUVECs treated with the S100A8/A9 heterodimer, but not with either homodimer, enhanced secretion of IL-6, ICAM-1, VCAM-1 and MCP1 via RAGE-ERK1/2-p39- NF-κB mediated pathway (25). S100A8/A9 is specifically expressed in neutrophils and macrophages, which have infiltrated an infarcted myocardium (71) and are specifically secreted upon interaction between phagocytes and activated endothelium (36). Indeed, S100A8/A9 is a critical component in the response to vascular injury as indicated by significant reductions in leukocyte accumulation and neointimal formation after femoral artery injury in S100A9-deficient mice compared to wild-type mice (18). Similarly, prolonged time to arterial thrombic occlusion has been observed in S100A9 knockout mice, with infusion of platelets expressing S100A8/A9 from wild type mice substantially reducing time to carotid occlusion after vascular injury, indicating that platelet-derived S100A9 is involved in the regulation of thrombosis (163).

The concentration of S100A8 and S100A9 in *in vitro* experiments appears to influence the inflammatory effects. For example, at low concentrations (up to 10ug/mL), S100A8/A9 has been found to promote angiogenic activity in vascular endothelial cells in both the homodimer or heterodimer form (92). As such, lower concentrations are believed to promote the growth of tumor cells and tumor cell migration (92, 153). Conversely, high concentrations have been found to have apoptotic effects on tumor cells, endothelial cells and other tissues (154). Indeed, Viemann et al. (2005) found that high concentrations of S100A8/A9 (200ug/mL) upregulated expression of genes involved in the promotion of platelet aggregation, endothelial permeability and inflammations in endothelial cells (151). A follow up study performed by this same lab found that S100A8/A9 induced apoptotic signaling in endothelial cells through caspase-dependent and -independent mechanisms (150).

S100A8/A9 and Exercise

Although the exact mechanisms are still under investigation, S100A8 and S100A9 have known roles in inflammatory disease pathologies. However, the effects of acute and chronic exercise on the secretion of these proteins are even less understood. Consistent with the inflammatory response to tissue injury, elevated plasma S100A8/A9 have been reported in response to high intensity or high volume exercise compared to resting levels (30). Substantial increases in circulating S100A8/A9 were observed after completion of a marathon (~96-fold), half marathon (~13 fold), 30-km run (~20-fold), multi-day military ranger course corresponding to 35% VO2max (~8 fold), and a VO2max test (~3 fold). This may be indicative of damage to the vascular endothelium caused by the stress of exercise resulting in microthrombi and subsequent activation of myeloid cells (30). These authors estimate the half-life of S100A8/A9 to be ~5 hrs following exercise. However, the participants in this study were all younger adults free of CVD, and thus further investigations into levels of S100A8/A9 in disease states associated with chronic inflammation are needed to fully understand the half-life of these proteins in other biological conditions.

Indeed, it appears that S100A8/A9 are playing a key role in the exercise-induced inflammatory response. Similar to the study by Fagerhol et al., another lab found a 6-fold increase in serum S100A8/A9 after a marathon run with levels remaining elevated for three hours after completion of the run and returning to resting levels after 24 hrs (111). Creatine kinase (CK) and C-reactive protein (CRP) levels in these marathon runners increased after completion of the run with the peak values occurring 24 hours after the run. Exercise intensity was also assessed, with an immediate increase in S100A8/A9 serum levels noted after a strenuous, but not after a moderate intensity exercise, followed by a significant increase in S100A8/A9 and CK concentrations in both groups one hour after the completion of exercise (111). Additionally, these authors found that eccentric exercise (downhill running), compared to concentric exercise, resulted in elevated serum S100A8/A9 even after 24 hrs and that this corresponded with elevated CK levels. These observed increases in S100A8/A9 following exercise were accompanied by increases in other inflammatory factors such as IL-6 and TNF-α, suggesting that S100A8/A9 are likely involved in an acute phase response immune system reaction that stimulates later increases in CRP (111).

Interestingly, marathon-induced increases in S100A8/A9 were 1.5-fold greater in highly trained individuals compared to their lower-fit counterparts, despite no differences in CK or CRP levels as a function of training status (111). This is contrary to other findings of a blunted exercise-associated stress response as a result of upregulated defense mechanisms in trained vs. untrained individuals. This response may also be tissue-specific, as we have found higher levels of S100A9 in the secretome of inactive individuals' CD34-/CD31+ CACs compared to that of endurance-trained individuals (88), although the levels in that particular study were cross-sectional in nature and assessed at rest without the added stimulus of exercise.

Mortensen et al. (2014) identified skeletal muscle as another source of S100A8/A9 during exercise. Three hours of cycle ergometer exercise at 60% VO2max upregulated S100A8 and S100A9 mRNA levels in human skeletal muscle by 5-fold and 3 hours of two-legged dynamic knee extensor exercise at 50% peak power output increased arterial plasma S100A8/A9 concentrations by 2-fold (113). This is the first study to note S100A8/A9 being released from skeletal muscle. The purpose of S100A8/A9 secretion from skeletal muscle or other tissues as a result of exercise is currently unknown. As there is a close relationship between activated inflammatory cells and circulating S100A8/A9, it has been suggested that it plays a role in the acute phase immune response associated with exercise (111, 113). Some authors speculate that it may serve a protective role against certain cancers, as S100A8/A9 have been found to induce apoptosis of tumor cell lines (113). Thus, it is possible that these proteins are serving to mediate both beneficial and detrimental effects of exercise on health. Of note, these studies investigating the effects of exercise on S100A8/A9 do so after extreme bouts of exercise. These levels far exceed what the average person performs on a daily basis, or what is recommended by the American College of Sports Medicine (ACSM) (107). Additionally, investigations into the secretion of S100A8/A9 from different tissues and/or myeloid cells as a function of exercise are minimal. As such, it is evident that more research is needed to understand the role of S100A8/A9 in exercise, health and disease.

Summary

One in every 4 deaths in the Unites States each year is caused by CVDs (45). As discussed above, traditional CV risk factors such as dyslipidemia, hypertension and diabetes account for only a fraction of events related to CVDs (112), emphasizing the need for investigations into more novel risk factors. Since the late 1990s, CACs have been identified as potential factors that may help account for some of these events as lower CAC number has been found to serve as a predictor of CV risk (7, 8, 47). Since then, many studies have investigated cell number as novel CV risk factor.

Although CAC is a broad term including any circulating cell with angiogenic potential, our lab has focused on isolating specific cells through surface markers in order to isolate a more defined population with known roles in angiogenesis. Several CAC subtypes have been used in experimental and/or clinical trials to examine the use of CACs in autologous cell transplants in the treatment of ischemic CVDs. Although promising in animal studies, these trials in humans have been met with mixed results (97, 120, 121, 143, 144). Collectively, the research in the CAC field has identified discordant findings in the use of CACs for therapies in human ischemic CVDs. Many believe this is a result of the autologous nature of these cells such that CACs from unhealthy individuals are being isolated, purified and re-introduced to the ischemic tissue of the donor. It is now understood that CACs from patient populations do not function as well as those from healthy individuals (76). As such, an emphasis has been placed on ways to enhance the functional capacity of CACs to be used for autologous cell therapies in order to improve the outcomes of this treatment (13, 58, 102).

As discussed above, endurance exercise training has been found to improve both CAC number and function. We have found that paracrine function of CACs is altered as a result of habitual endurance exercise, with secreted factors from inactive individuals exhibiting lower angiogenic capacity compared to endurance-trained individuals. These findings and other research supporting the theory that aerobically fit individuals have higher functioning cells have led us to use CACs from this population as a model of optimal CAC function and means of comparison when studying CACs from CVD patients. As it is now generally accepted that CACs function predominantly through paracrine actions, it is critical to understand the factors being secreted by CACs as a function of health and fitness status that may help or hinder angiogenesis if autologous CAC therapy is to be successfully administered in the future.

Chapter 3: Chronic endurance exercise affects paracrine action of CD31+ and CD34+ cells on endothelial tube formation

The following article was published in the American Journal of Physiology, Heart and Circulatory Physiology 2015 Aug 1;309(3):H407-20

Chronic endurance exercise affects paracrine action of CD31+ and CD34+ cells on endothelial tube formation

Rian Q. Landers-Ramos[1], Ryan M. Sapp[1], Nathan T. Jenkins[2], Anna E. Murphy[1], Lucile Cancre[1], Eva R. Chin[1], Espen E. Spangenburg[1], James M. Hagberg[1]

[1]Department of Kinesiology, School of Public Health, University of Maryland College Park, College Park, MD

[2]Department of Kinesiology, University of Georgia, Athens, GA

Correspondence: James M. Hagberg, Ph.D., 2134E SPH Bldg, University of Maryland, 255 Valley Dr, College Park, MD 20742-2611. 301-405-2487 (TEL), 301-405-5578 (FAX). **hagberg@umd.edu**

Running Title: Exercise and paracrine effects of CACs

ABSTRACT

We aimed to determine if chronic endurance exercise habits affected redox status and paracrine function of CD34+ and CD34-/CD31+ circulating angiogenic cells (CACs). Subjects were healthy, nonsmoking, men and women aged 18-35 yrs and categorized by chronic physical activity habits. Blood was drawn from each subject for isolation and culture of CD34+ and CD34-/CD31+ CACs. No differences in redox status were found in any group across either cell type. Conditioned media (CM) was generated from the cultured CACs and used in an *in vitro* HUVEC-based tube assay. CM from CD34+ cells from inactive individuals resulted in tube structures that were 29% shorter in length ($P<0.05$) and 45% less complex ($P<0.05$) than the endurance-trained group. CD34-/CD31+ CM from inactive subjects resulted in tube structures that were 26% shorter length ($P<0.05$) and 42% less complex ($P<0.05$) than endurance-trained individuals. Proteomics analyses identified S100A8 and S100A9 in the CM. S100A9 levels were 103% higher ($P<0.05$) and S100A8 was 97% higher in the CD34-/CD31+ CM of inactive subjects compared to their endurance-trained counterparts with no significant differences in either protein in the CM of CD34+ CACs as a function of training status. Recombinant S100A8/A9 treatment at concentrations detected in inactive subjects' CD34-/CD31+ CAC CM also reduced tube formation ($P<0.05$). These findings are the first, to our knowledge, to demonstrate a differential paracrine role in CD34+ and CD34-/CD31+ CACs on tube formation as a function of chronic physical activity habits and identifies a differential secretion of S100A9 by CD34-/CD31+ CACs due to habitual exercise.

INTRODUCTION

Cardiovascular disease (CVD) and the associated dysfunction of the vasculature is the leading cause of death in the developed countries (45). Studies suggest that the repair and maintenance of the vascular endothelium is critically dependent on the number and function of circulating angiogenic cells (CACs) (4, 147, 172). CAC is a broad term for subgroups of peripheral blood mononuclear cells with angiogenic properties that stimulate vascular repair and contribute to neovascularization. CD34 is the most commonly described cell surface marker on CACs that are known for their progenitor cell properties (103). CD31 is a cell surface marker most commonly found in mature endothelial cells, but has also been identified in circulating cells with angiogenic properties (64, 75, 76, 78, 84). Although not as commonly studied as CD34+ CACs for therapeutic neovascularization, CD34-/CD31+ CACs have been found to exhibit similar angiogenic potential as CD34+ CACs in terms of improving mouse hind-limb ischemia, emphasizing the importance of studying non-progenitor cell lines which may also be involved in angiogenesis (78). Indeed, cells characterized by both markers have been previously found to exhibit angiogenic characteristics *in vitro* (4, 53, 75, 125) and *in vivo* (79, 95, 103).

Originally believed to exert their reparative functions through direct incorporation into the endothelium (4), recent evidence indicates that CACs accomplish their pro-angiogenic effects through paracrine mechanisms. Specifically, CACs are thought to secrete factors that elicit actions on the pre-existing endothelium and/or signal other circulating cells to home to the endothelium (79, 81, 125, 145, 175). Recently, Hynes et al. (2011) demonstrated that secretome from cultured CACs successfully treated a porcine model of myocardial infarction, suggesting that paracrine factors secreted by CACs may be more important for vascular repair than the cells alone (54). Urbich et al. found that CACs expressed and released a number of different factors that assist with the growth of mature endothelial cells to accelerate revascularization in ischemic tissues (145). However, CD34+ or CD34-/CD31+ CAC paracrine actions and their susceptibility to modulation by lifestyle factors (e.g., physical activity) have received minimal investigation.

Physical activity is associated with improvements in CV health, including attenuation of many classic CVD risk factors (65, 98) and improvements in endothelial function (22, 133). Our lab and others have previously shown that endurance exercise training increases CAC number and enhances intracellular redox balance in younger and older adults compared to their sedentary counterparts (63, 66, 94, 146-148, 166). Hoetzer et al (2007) found that migratory activity of sedentary individuals' CACs improved by ~50% after 12 weeks of exercise training(50). We previously documented that CACs from young sedentary but otherwise healthy individuals exhibit an unfavorable balance between reactive oxygen species (ROS) and nitric oxide (NO) compared to their endurance-trained counterparts (63, 66). It is generally accepted that CACs with less than optimal function cannot contribute properly to vascular repair or neoangiogenesis, thus hindering their therapeutic use (35).

Previous studies have examined the effects of endurance exercise habits on CAC function through measures such as migration and adhesion (50, 146), but the paracrine function of CD34+ and CD34-/CD31+ CACs on angiogenesis has not been considered. The purpose of this study was to determine whether there is a differential response in paracrine function of CD34+ and CD34-/CD31+ CACs as a result of chronic endurance exercise habits in younger men and women and to identify differences in CAC intracellular redox pathways that may be eliciting angiogenic or anti-angiogenic actions. We hypothesized that both CD34+ and CD34-/CD31+ CACs from endurance-trained individuals will have enhanced paracrine function that will result in superior angiogenic capacity compared to inactive individuals and that this will be linked to a healthier redox balance in the endurance-trained group. A secondary aim of our study was to identify proteins present in the secretome of CD34+ and CD34-/CD31+ CACs and to determine whether these proteins are differentially expressed as a function of chronic exercise training status.

METHODS

Ethical Approval

The University of Maryland College Park Institutional Review Board approved all study procedures and subjects provided written informed consent. The study procedures conformed to the standards set by the Declaration of Helsinki.

Screening

Subjects in this study were healthy, nonsmoking, men and women aged 18-35 yrs with no history of CV or metabolic disease. Potential subjects were initially screened by telephone or email, and reported to the laboratory following an overnight fast for a screening visit to verify eligibility. Subjects were categorized based on their reported physical activity over the last 5 years and confirmed via VO_2max. Specifically, inactive subjects (n=12; 5 women and 7 men) reported performing ≤20 min endurance exercise on ≤2 days/week. The active group (n=15, 5 women and 10 men) reported performing ~4 hours/week of low to moderate intensity activity and the endurance-trained group (n=14, 9 women and 5 men) reported performing >4 hours/week of moderate to high intensity endurance exercise. Groups were matched for age and body mass index (BMI). Exclusion criteria were as follows: systolic blood pressure ≥130 mm Hg, diastolic blood pressure ≥90 mm Hg, serum total cholesterol ≥200 mg/dl; low-density lipoprotein-cholesterol ≥130 mg/dl; high-density lipoprotein-cholesterol ≤ 35 mg/dl; fasting glucose ≥100 mg/dl. Women were all tested during the early follicular phase of their menstrual cycle.

Maximal Graded Exercise Test, Body Composition and Blood Sampling

A screening blood sample was obtained for assessment of fasting serum triglyceride (TG), lipoprotein lipids, and glucose (Quest Diagnostics, Baltimore, MD).

Height, weight, seated blood pressure and BMI were measured, and body composition was assessed using the 7-site skinfold procedure (62). Maximal oxygen consumption (VO_2max) was assessed using a constant-speed treadmill protocol with 2–3% increases in incline every 2 min until exhaustion. The treadmill speed was based on the subject's experience, typical run speed, and heart rate such that VO_2max was achieved within 6–12 min. Pulmonary ventilation and expired gas concentrations were analyzed in real time using an automated computerized indirect calorimetry system (Oxycon Pro, Viasys). VO_2 was considered maximum if a plateau was achieved (increase in VO_2 of < 250 ml/min with increased work rate). In the absence of a clear plateau, tests had to meet at least two of the following secondary criteria: a respiratory exchange ratio > 1.10, a rating of perceived exertion >18, and a peak heart rate within 10 beats/min of the age-predicted maximum. On the testing day for blood sampling for CACs, the subjects reported to the laboratory in the morning after an overnight (~12 hr) fast. Endurance-trained and active subjects performed their normal exercise routine 16-24 hours prior to the blood sampling. A sample of 50mL of blood was drawn using EDTA-tubes (Becton Dickinson) for isolation of CD34+ and CD34-/CD31+ CACs.

Immunomagnetic Cell Separation

Peripheral blood mononuclear cells (PBMCs) were isolated from the venous blood samples using density gradient centrifugation (Ficoll, GE Healthcare). The CD34+ fraction was purified using multiple rounds of immunomagnetic cell separation according to the manufacturer's instructions (EasySep® Immunomagnetic Cell Separation Kits, STEMCELL Technologies) using an Ab specific for CD34. CD31+ cells were selected from the CD34- fraction of cells and purified as described above using and Ab specific for CD31 (hereby referred to as CD34-/CD31+). Multiple flow cytometry analyses in our lab have resulted in a CD34+ cell isolation purity of 52 ± 3% in the positively-selected fraction (compared to the 0.1% in total PBMCs prior to selection (Figure 3.1A and 3.1B) and virtually no detectable CD34$^+$ cells (<0.1%) in the CD34-depleted fraction (Figure 3.1C). This isolation approach has been published previously by our lab (63, 64) and the purity of our isolation is equivalent to or greater than other published results also using non-mobilized blood (4, 6, 41, 128, 160). The different surface antigen combinations were chosen on the basis of previous research indicating the involvement of stem/progenitor (i.e., CD34+), and endothelial antigen-expressing (i.e. CD31+) PBMC subsets originating from bone marrow or the vessel wall in the maintenance and repair of the vascular endothelium (6, 23, 41, 75, 79, 128).

CAC Culture, Conditioned Media, and Angiogenesis Assay

CD34+ and CD34-/CD31+ CACs were re-suspended in un-supplemented endothelial growth medium free of growth factors or serum, (EBM-2, Lonza) with 1% Antibiotic-Antimycotic (Invitrogen) each at a density of 100,000 cells per well. Cultures were maintained for 48 hrs in a humidified incubator at 37°C and 5% CO_2. After incubation, the conditioned media (CM) from all wells of the same cell type was withdrawn, combined into one tube and clarified by spinning at 2,500xg for 20 min to remove cells and debris from the medium. For the angiogenesis tube formation assay (2, 19, 39, 125), culture plates were coated with Reduced-Growth Factor Matrigel (BD Biosciences) and the matrigel was left to solidify for 30 min at 37°C and 5% CO_2. Under these *in vitro* conditions, HUVECs will form multi-cell cords, which serve as a global indicator of the angiogenic cascade. Each condition was performed in duplicate and each well contained 20,000 human umbilical vein endothelial cells (HUVECs) and equal volumes of CM from either CD34+ or CD34-/CD31+ CACs. Control wells were prepared with a similar amount of fresh EBM-2. The average HUVEC passage used in the angiogenesis assay for endurance-trained, active, and inactive subjects was 4.4±0.4, 4.6±0.3 and 4.2±0.3, respectively (n.s.). These plates were cultured for 16 hrs at 37^{o}C and 5% CO_2. The cultures were then visualized under a light microscope and 5 random images were photographed per well. These images were then coded and blindly assessed for HUVEC tube length and complexity (2, 9, 67) by two individuals. Tube length of each segment was quantified using ImageJ and averaged over the total number of images. Complexity was quantified as the number of nodes present, with a node being a site with $\geq$3 branching points. Results are presented as each condition normalized to the basal (EBM-2) condition to control for daily variability in HUVEC growth and passage number with each assay. Average readings of the two individuals were used for statistical analyses.

Measurement of Intracellular NO and ROS

These experiments were performed on freshly isolated cells in duplicate as we have described previously (63, 64), with minor modifications. Briefly, 1.5×10^5 cells were stained with 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate for determination of intracellular NO levels or 2 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for determination of intracellular ROS levels (Molecular Probes). 4',6-diamidino-2-phenylindole (DAPI; 750 ng/ml) was used to identify cell nuclei (Molecular Probes). Cells were incubated with fluorescent dyes in a final volume of 150 μl serum-free PBS for 30 min at 37°C. NO and ROS fluorescence were quantified using a fluorescent microplate reader (Biotek) using excitation and emission filters of 488 and 535 nm, respectively. DAPI fluorescence was measured using excitation and emission filters of 355 and 460 nm, respectively. NO and ROS fluorescence values were divided by DAPI fluorescence values to normalize for cell number. All fluorescent probes were validated using positive and negative controls as described previously by our lab (63, 64). Briefly, we observe several fold increases in intracellular H2DCF-DA signal in the presence of *3-Morpholinosydnonimine* (200 μM, Figure 3.4A). Pretreatment with PEG-catalase (50U/mL) reduces H2DCF-DA signal by nearly 40% after exposure to hydrogen peroxide (500 μM, Figure 3.4B). Additionally, we observed several fold increases in DAF-FM signal using NO donor, *Diethylenetriamine NONOate (*50 μM, Figure 3.5A) or *3-Morpholinosydnonimine* (20 μM, Figure 3.5B) and substantial reduction in DAF-FM signal with L-NAME treatment (300 μM, Figure 3.5B). Intra-assay coefficients of variation for ROS and NO were 5.4% and 4.8%, respectively.

Assessment of Gene Expression by RT-PCR

RNA was extracted from freshly isolated CACs using the TriZol reagent, DNase treated using TURBO DNA-free™ Kit (Life Technologies), and reverse transcribed to cDNA. Expression of angiogenic [endothelial nitric oxide synthase (eNOS)], and pro-oxidant [NADPH oxidase subunits gp91^{phox} and p47^{phox}] genes were assessed as previously described (66) to confirm our hypothesis that ROS and NO were mediating differences in CAC paracrine function. Additional gene targets include S100A8 (Forward primer: 5’-AGCCCTGCATGTCTCTTGTC-3’, Reverse primer: 5’-ACGTCTGCACCCTTTTTCCT-3’) and S100A9 (Forward primer: 5’-TCATCATGCTGATGGCGAGG-3’, Reverse primer: 5’-

CCTGGCCTCCTGATTAGTGG-3’), which were assessed to confirm that each CAC subtype expressed the mRNA to produce the S100A8 and S100A9 proteins identified in our CM.

Mass Spectrometry, Western Blot Analysis and S100A8 and S100A9 Expression

Previously clarified CM from CD34+ and CD34-/CD31+ cells was divided into aliquots and stored at -80^{o}C until further analyses. Proteomics techniques were used to determine proteins present in the CD34+ and CD34-/CD31+ CM from of each endurance-trained and inactive groups. Spectral counts for n=1 per group were used as an initial non-labeled method providing semi-quantitative data (99). Subsequently, these findings were quantitatively confirmed using immunoblotting analyses on n=12 per group. Media was concentrated using Amicon Ultra 0.5 3kD centrifugal filter devices (Millipore). The soluble proteins in the supernatant were subjected to digestion with trypsin Lys-C. Samples were analyzed using a ThermoFinnigan LTQ Orbitrap XL mass spectrometer, and matched to the UniProtKB database. Western blot analyses were used to assess differences in two identified S100 proteins with specific antibodies for S100A8 (R&D Systems) and S100A9 (Santa Cruz Biotech). Membranes were washed and the incubated with horseradish peroxidase (HRP)- conjugated anti-mouse IgG (Cell Signaling; 1:1000) or HRP-conjugated anti-goat IgG (Novus; 1:5000) secondary antibodies. Blots were developed using Super Signal ECL reagents (Thermo Scientific) and bands were visualized and quantified using Chemi-Doc Imaging System and software (BioRad). Values were normalized to 100,000 cells/well used to generate the CM. Recombinant human S100A8/A9 complex (R&D Systems) was used in western blots to establish a standard curve based on concentrations reported in the literature (92, 151) and to quantitatively assess levels present in the CM of each CAC subtype.

Recombinant S100A8/A9 treatment

To confirm the direct effects of S100A8 and S100A9 on HUVEC tube formation, the concentrations and proportions estimated in the CM of the inactive subjects' CD34-/CD31+ CACs were used in an angiogenesis assay. As estimated, 7.18 µg/mL of recombinant human S100A8 (ProSpec Bio) and 3.06 µg/mL of recombinant human S100A9 (Life Technologies) were added to a HUVEC-based tube formation assay and compared to the positive control prepared with EBM-2 and vehicle control. In these experiments, each condition was assessed in samples collected from six independent cell culture wells from multiple culture plates collected on different days. All experiments were conducted on cells from the same passage number (P4). As described above, each well contained 20,000 HUVECs. The plates were cultured for 16 hrs at 37^{o}C and 5% CO_2. The cultures were then visualized under a light microscope and 5 random images were photographed per well.

Statistics

Statistical analyses were completed using IBM SPSS Statistics 21. Assumptions of homoscedasticity and normality were verified for all outcome measures. Data were analyzed using multivariate ANOVA with pairwise comparisons where appropriate. t-tests were used for western blot analyses. Statistical significance was accepted at $P \leq 0.05$. Values are expressed as mean ± standard error of the mean.

RESULTS

Subject characteristics (Table 3.1.)

All subject groups (n= 14 endurance trained, n=15 active and n=12 inactive) were of the same age and BMI. Training status was confirmed through VO_2max with the endurance-trained subjects having 15% greater VO_2max compared to active and 40% greater VO_2max compared to inactive subjects ($P<0.05$). Active subjects had 30% higher VO_2max than inactive ($P<0.05$). Despite the three groups being matched for BMI, inactive subjects had significantly higher body fat percentage than both the active and endurance-trained subjects ($P<0.05$). Importantly, subjects were all matched for the majority of cardio-metabolic risk factors including systolic blood pressure, glucose, cholesterol, TG, and lipoprotein lipids.

Angiogenic tube formation with conditioned media

CD34+ cells. Conditioned media (CM) from a purified population of CD34+ CACs (Figure 3.1) resulted in 29% lower tube length ($P<0.05$) and 45% lower complexity ($P<0.05$) in inactive subjects compared to endurance-trained subjects, and 14% lower length ($P<0.05$) and 20% lower complexity ($P=0.05$) in active subjects compared to endurance-trained subjects (Figure 3.2A and 3.2B). CD34+ CM from inactive subjects produced lower HUVEC length (n.s.) and complexity ($P=0.05$) than CD34+ CM from active subjects (15% and 26%, respectively; Figure 3.2A and 3.2B). Representative images from each condition can be found in Figure 3.3. CM from both endurance-trained and active subjects CD34+ CACs did not result in statistically different tube length or complexity ($P>0.05$) compared to basal conditions whereas CM from inactive individuals CACs resulted in significantly lower length and complexity compared to basal ($P<0.05$).

CD34-/CD31+ cells. HUVEC tube length was 26% lower ($P<0.05$) and complexity was 42% lower ($P<0.05$) with CM from CD34-/CD31+ CACs of inactive subjects compared to endurance-trained individuals. CD34-/CD31+ CM from endurance-trained subjects was not significantly different in terms of tube length and complexity than CD34-/CD31+ CM from active subjects (Figure 3.2C and 3.2D). Although not statistically significant, CM from CD34-/CD31+ CACs of inactive subjects resulted in 16% shorter tube length and 28% less complexity than active subjects (Figure 3.2C and 3.2D). There was no significant effect of sex on tube formation for either CD34+ or CD34-/CD31+ CM ($P>0.05$). Representative images from each condition can be found in Figure 3.3. The CD34-/CD31+ CM from endurance-trained and active subjects' CACs did not result in significantly different tube length or complexity ($P>0.05$) compared to basal conditions, whereas CD34-/CD31+ CM from inactive individuals' CACs caused significantly lower length and complexity compared to basal ($P<0.05$).

Intracellular ROS and NO

To determine if the differences in CM-induced tube formation were associated with differences in intracellular redox pathways, we assessed intracellular ROS and NO levels using previously validated techniques (Figures 3.4A-B and Figure 3.5A-B) and a purified population of cells (Figure 3.1). There were no significant differences in intracellular ROS levels between endurance trained, active or inactive subjects' CD34+ CACs (Figure 3.4C) or CD34-/CD31+ CACs (Figure 3.4D). In addition, no significant differences between intracellular NO levels were detected between CD34+ CACs from endurance trained, active and inactive subjects (Figure 3.5C) or CD34-/CD31+ CACs

(Figure 3.5D). There was no significant effect of sex on intracellular ROS or NO levels for either cell type.

Gene Expression

CD34+ cells. There were no significant effects of training status on CD34+ eNOS (Figure 3.6A) or p47^{phox} or gp91^{phox} mRNA expression (Figures 3.6B and 3.6C). There was no significant effect of sex on expression of any of our genes of interest in CD34+ CACs.

CD34-/CD31+ cells. eNOS gene expression was not statistically different in inactive subjects compared to active and endurance-trained subjects (Figure 3.6D). There were no significant differences between groups for p47phox or gp91phox gene expression in CD34-/CD31+ CACs (Figure 3.6E and 3.6F). There was no significant effect of sex on gene expression for any of our targets for CD34-/CD31+ CACs.

Detection of Proteins in Conditioned Media

In an attempt to identify secreted paracrine factors from CACs, conditioned medium from CD34+ and CD34-/CD31+ cells was analyzed by mass spectrometry. Two targets from the S100 family of proteins, S100A8 and S100A9, were identified in the secretome of both CD34+ and CD34-/CD31+ cells (Figure 3.7). Due to our detection of S100A8 and S100A9 in the CM of each CAC sub-type, we sought to confirm that CACs express these genes. S100A8 and S100A9 were expressed in both CAC sub-types (Figure 3.8). In CD34+ CACs, there were no significant differences between groups in S100A8 mRNA or S100A9 mRNA (Figure 3.8A and 3.8B). S100A8 and S100A9 mRNA in CD34-/CD31+ CACs were also not significantly different as a function of training status (Figure 3.8C and 3.8D).

The S100A8 and S100A9 proteins are known mainly for their role in inflammation but their role in the regulation of angiogenesis is not well understood, especially as a secreted factor from our cells of interest. As such, we sought to determine if differences existed in the levels of these proteins present in CAC conditioned media as a result of chronic exercise training status. Immunoblotting for S100A8 and S100A9 in the conditioned media of CD34+ cells did not show significant differences between endurance-trained and inactive subjects (Figure 3.9A and 3.9B). There was also no significant sex-related difference in the content of either protein in CD34+ CM. In CD34-/CD31+ cells, there were no significant differences in S100A8 content between endurance trained and inactive subjects ($P=0.08$; Figure 3.9C). However, S100A9 content was significantly higher in CD34-/CD31+ conditioned media of inactive subjects compared to endurance-trained subjects ($P<0.05$; Figure 3.9D). There was no significant effect of sex on S100A8 or S100A9 content in CM from CD34-/CD31+ CACs.

Using recombinant human S100A8/A9, a standard curve was established using typical concentrations employed in the literature (92, 151) to allow us to estimate total content of each protein that the HUVEC cells were exposed to in a subset of remaining CM samples (Figure 3.10A). For this experiment, we used n=3 samples from the inactive groups' CD34-/CD31+ CACs to estimate the concentrations of each protein that could potentially be responsible for depressed tube formation. As no differences in either S100A8 or S100A9 were noted in the CM of CD34+ CACs, we used n=2 inactive CM and n=1 endurance trained subjects' CM to estimate the concentrations of each of these proteins. The total amount of S100A8 was estimated to be 8.69±4.3 μg/mL and S100A9 was estimated to be 16.97±4.6 μg/mL in CD34+ cell CM (Figure 3.10B). CM for CD34-

/CD31+ CACs contained approximately 7.18±0.28 µg/mL of S100A8 and 3.06±0.95 µg/mL of S100A9 (Figure 3.10C). We next calculated the proportion of S100A8/S100A9 and found that CD34+ CM had an A8/A9 ratio of 0.513, while CD34-/CD31+ CM contained an A8/A9 ratio of 2.343 (Figure 3.10B and 3.10C).

Recombinant human S100A8 and S100A8 treatment

Recombinant human S100A8 and S100A9 were added to a HUVEC-based tube formation assay in the concentrations and proportions that were estimated to be present in the CD34-/CD31+ CM of inactive subjects (7.18 µg/mL of S100A8 and 3.06 µg/mL of S100A9). Compared to the basal control, addition of S100A8 and S100A9 resulted in an average of 18% lower tube length and 28% lower complexity ($P<0.05$ for each; Figure 3.11).

DISCUSSION

Paracrine actions are believed to be the major mechanism through which CACs exert their angiogenic properties on the pre-existing endothelium, but it was previously unknown whether regular endurance exercise affected these actions. In this study we demonstrate that conditioned media generated from inactive subjects' CD34+ and CD34-/CD31+ CACs depresses paracrine-mediated tube formation. These data strongly suggest that endurance exercise training-mediated alterations in CAC paracrine activity influence angiogenesis. Other studies have demonstrated the role of paracrine signaling of CACs in diseased states (54, 125, 174). Our data provide further support for a paracrine role for CD34+ and CD34-/CD31+ CACs on angiogenesis in younger, healthy individuals. More importantly, to our knowledge, we are the first to report of a differential paracrine role in CD34+ and CD34-/CD31+ CACs on HUVEC-based tube formation as a function of chronic physical activity habits.

Based on previous research from our lab (63), we hypothesized that differences in intracellular ROS and NO concentrations as a result of exercise-training status would at least partially explain our observed differences in paracrine function in both cells types. Surprisingly, the present data suggest that intracellular ROS and NO are not playing a major role in regulating the differential paracrine responses observed. We found no significant effects of training status on intracellular NO or ROS levels or in mRNA expression for eNOS as well as $p47^{phox}$ and $gp91^{phox}$ for either cell type. Although some studies have found an effect of endurance exercise on improved redox balance in both healthy (63, 161) and diseased populations (33, 131, 136), others have found improvements in endothelial function with endurance exercise independent of changes in oxidative stress (98). It is important to note that these previous studies have focused mainly on endothelial progenitor cells (EPCs), which share some of the same characteristics as the two CAC subtypes that we studied, but generally they express both the progenitor cell marker (CD34) and the endothelial marker (CD31 or VEGFR2) simultaneously and are negative for CD45. As these cells can have very different properties, caution must be used when comparing studies using different cell types as a result of selection or culture conditions. Importantly, our previous cross-sectional studies included only men (63, 66), whereas in the current study we included both men and women, potentially accounting for the different findings in the current study compared to our previous work. Recently, Guhanarayan et al. (2014) found that 10 days of reduced physical activity decreased intracellular NO in CFU-Hill CACs, but they observed no differences in intracellular NO levels in freshly isolated CD34+ CACs similar to those investigated in the present study (40). Additionally, they found no significant changes in NO or ROS-related gene expression with reduced physical activity in either CAC type. Together, these data suggest that the effects of intracellular ROS and NO may be specific to certain populations of CACs and emphasize the need for future investigations.

In an attempt to elucidate potential contributors to our observed differences in tube formation, we utilized mass spectrometry analyses to assess the secreted protein content of the conditioned media. We identified two members of the S100 family of calcium-modulated proteins, S100A8 and S100A9, in the secretome of both CD34+ and CD34-/CD31+ CACs. Further, we confirmed that both CAC sub-types expressed mRNA species specific for both of these proteins, indicating that both cell types possess the necessary molecular machinery to produce the proteins. As our observed differences in tube formation appeared to be a graded effect, we focused on the two extreme groups, endurance-trained and inactive, for protein analyses of our conditioned media. We found that the protein content of S100A9 was twice as high in the CD34-/CD31+ CM of inactive subjects compared to their endurance-trained counterparts. These findings are the first, to our knowledge, to demonstrate differential secretion of S100A9 by CD34-/CD31+ CACs as a function of an individual's exercise-training status. Although not significant, S100A8 was also roughly twice as high in the CD34-/CD31+ CM from inactive subjects compared to endurance-trained subjects. S100A8 and S100A9 proteins are abundantly expressed in both myeloid and vascular cells (5) and are most commonly known for their role in regulating inflammatory processes (5, 27, 153). Several studies have linked systemic concentrations of the S100A8/S100A9 heterodimer complex to CVDs (5) with plasma levels predicting future CV events in middle-aged healthy individuals (17, 18). Thus, the higher levels of S100A9 in inactive individuals are consistent with these previous findings indicating these proteins as a risk factor for CVD. Croce et al. (2009) demonstrated the enhanced stability of these proteins when functioning as a heterodimer complex (18). Given the biology of the S100A8/A9 complex, it is possible that our S100A8 findings in CD34-/CD31+ CM paired with the significant differences in S100A9 may be physiologically relevant despite the lack of statistical significance.

In our hands, we found that our CM from both cell types contained less than 20µg of S100A8 and S100A9. Most investigations assessing the *in vitro* effects of S100A8 or S100A9 utilize concentrations between 1-200 µg/mL (5, 92, 151). Thus, our CM content is on the low end of the established *in vitro* range of S100A8 or A9 concentrations.

Defining the concentrations of S100A8 or A9 is important since previous publications have shown concentration dependent effects on angiogenic function (153). In additions, both proteins are able to activate signaling in a homodimer, heterodimer or heterotetramer form (153), thus adding more complexity to their mechanism of action. When the proportion of S100A8/S100A9 was calculated in the CM there was a greater proportion of S100A8/S100A9 in the CD34-/CD31+ CM compared to that of the CD34+ CM. These results suggest that CM from the different cells types are fundamentally different from each other even though both showed similar effects on the HUVEC cells. The differences in the ratios of these proteins in the CM of the two different cell types may provide one explanation as to why we observed differences in these proteins as a function of training status in the CD34-/CD31+ CM but not in the CD34+ CM. This also suggests that other secreted factors may better explain our observed differences in CD34+ CM-mediated HUVEC tube formation as a function of training status.

As we found significantly greater levels of S100A8/A9 in the CD34-/CD31+ CM of inactive individuals compared to endurance-trained individuals, we sought to empirically test the effects of these proteins on HUVEC tube formation. We added recombinant human S100A8 and S100A9 using the estimated concentrations of each of these proteins that were present in the CM of CD34-/CD31+ CACs. We found that HUVEC length was approximately 18% lower and complexity was 28% lower in this condition compared to the positive control. Importantly, these findings confirm the role of each of S100A8 and S100A9 in the discovered concentrations as a depressor of HUVEC tube formation.

As only a small percent of the population are high-level endurance athletes, a strength of our study is the inclusion of a regularly active group that met the guidelines for physical activity without being defined as “highly trained” or “elite” as this group is more applicable to the general population (107). In the current study, we observed a graded effect of exercise with CM from endurance-trained athletes contributing best to tube formation and CM from inactive subjects having a negative effect on tube formation. We observed no significant differences in tube formation between the active and endurance-trained groups in the CD34-/CD31+ CM conditions suggesting that the range of physical activity influences the function of the CACs. In this study, we only examined levels of S100A8 and S100A9 in the CM from the two extreme groups, so future studies should also follow up on habitually physically active individuals to determine the concentrations on these proteins being secreted by their CACs.

This study is also unique in that it includes data from both young men and women. We did not detect significant differences between any variable measured due to sex although we acknowledge that with such a small sample of women the ability to detect statistically significant differences is low. Regardless, very little research in the CAC field focuses on pre-menopausal women, possibly due to the difficulty controlling for menstrual cycle status, as estrogen and other female sex hormones have known effects on CAC function (59, 60, 104). In our study, we attempted to control for the effects of estrogen throughout the menstrual cycle by testing all women during the follicular phase when estrogen is typically at its lowest levels (140). We acknowledge that there are individual fluctuations in estrogen levels throughout the menstrual cycle, and we did not directly measure estrogen levels, which could have contributed to variations in some of our findings. It is also important to mention that our subject groups were heterogeneous with respect to representation of men and women, with the endurance-trained groups being comprised of proportionally more women compared to the other groups. However, we do feel that it is an important contribution to the literature to include this population both as a comparison to men and to begin to learn more about the health and function of CACs from pre-menopausal women.

Various sub-populations of CACs have been used in autologous cell therapies as a means for treating ischemic CVD. It is now well accepted, and our results support, that CACs work through paracrine functions (38, 78, 126, 160). A sedentary lifestyle is associated with future CVD risk through diseases such as obesity and Type II Diabetes. In the absence of lifestyle changes, it is possible that differences in the secretion of these proteins in sedentary but otherwise young, healthy individuals will become more exacerbated with age and progression of CVD. As such, our findings of greater S100A8 and A9 secretion by CD34-/CD31+ CACs of young, inactive individuals provide rationale for future studies to determine whether the dysfunctional properties of CACs from CVD patients are linked to altered S100A8 and A9 secretion.

Limitations

We were limited in the number of functional assays that we could perform due to the low overall amount of CM that was generated from our isolated cells. Due to this limitation, we performed an *in vitro* assay that has been widely used as a global readout of the entire angiogenesis cascade. We acknowledge that the use of the HUVEC-based angiogenesis tube formation assay does not individually assess all the critical regulatory steps during angiogenesis. Future studies should include measures such as HUVEC proliferation and migration as a complement to the angiogenesis assay to more comprehensively assess angiogenic activity in endothelial cells. Additionally, further research on the different proportions of S100A8 and S100A9 as a function of training status would provide more understanding into function of these proteins. In the current study we used recombinant S100A8 and S100A9 to reproduce the depressed tube formation observed when using CD34-/CD31+ CM from inactive subject's CACs. We acknowledge that other secreted factors are potentially contributing to our observations. Future experiments should employ a loss of function approach to confirm that S100A8 and S100A9 are mediating the differences in tube formation or determine whether other secreted proteins present in the CM are affecting HUVEC function as well. Neutrophils and other myeloid cells are major producers of S100A8 and S100A9. Our CAC isolations yield a purity of approximately 50% and therefore, we cannot exclude the possibility of other cells contributing to the levels of S100A8 and S100A9 in our CM. It is unlikely that one individual cell type would make up the majority of the other 50% consistently across training groups. Rather, this is more likely comprised of a heterogeneous mix of cell types. As such, it is probable that the differences observed in our study are predominantly due to each of the purified CAC subtypes, although further characterization of other cell types present after CAC purification are necessary to confirm this. Furthermore, rates of cell proliferation over the culture period among different training groups should be considered in future work to confirm that the observed differences are due to greater secretion of S100A8 and S100A9 and not a greater number of cells.

All measures in this study were taken under basal cell conditions, which is not always representative of the *in vivo* environment. Under resting conditions these cells are less active and many studies have found that pharmacological challenges such as LPS or

PMA stimulate cytokine/growth factor secretion from various cell types (101). We have previously shown that *in vivo* consumption of a high fat meal alters the intracellular ROS levels of CACs (64); thus it is our belief that a similar stimulation may also alter the paracrine profile of CACs. Follow-up experiments investigating how an *in vivo* or *in vitro* challenge affect CAC paracrine functions will be necessary to fully elucidate the paracrine and autocrine behaviors of these cells.

Conclusions

In summary, we demonstrate, for the first time, a differential effect on tube formation when HUVECs were cultured with CM from either CD34+ or CD34-/31+ CACs of endurance-trained athletes, active or inactive individuals. CM from inactive subject’s CACs appears to produce an inflammatory effect that depresses tube formation and this effect is not evident with increased chronic exercise behaviors. We found that pro-inflammatory S100A9 secreted by CD34-/CD31+ CACs is significantly higher in the CM of inactive individuals compared to their endurance-trained counterparts and culture with recombinant S100A8 and S100A9 in concentrations estimated to be present in the CD34-/CD31+ CM significantly reduced HUVEC tube formation. These results support the possibility that increases in secreted S100A8/A9 in inactive individuals are contributing to the reduced HUVEC tube formation associated with CM from CD34-/CD31+ CACs.

COMPETING INTERESTS

No competing interests are reported.

AUTHOUR CONTRIBUTIONS

RQL, NTJ, ERC, EES and JMH conceived and designed the research experiments. RQL, RMS, AEM, and LC performed the experiments and collected the data. Analysis and interpretation of data was performed by RQL, NTJ, ERC, EES, and JMH. RQL drafted the manuscript; RMS, NTJ, AEM, LC, ERC, EES, and JMH edited and revised the paper and provided important intellectual content. All authors approved the final version of the manuscript.

ACKNOWLEDGEMENTS

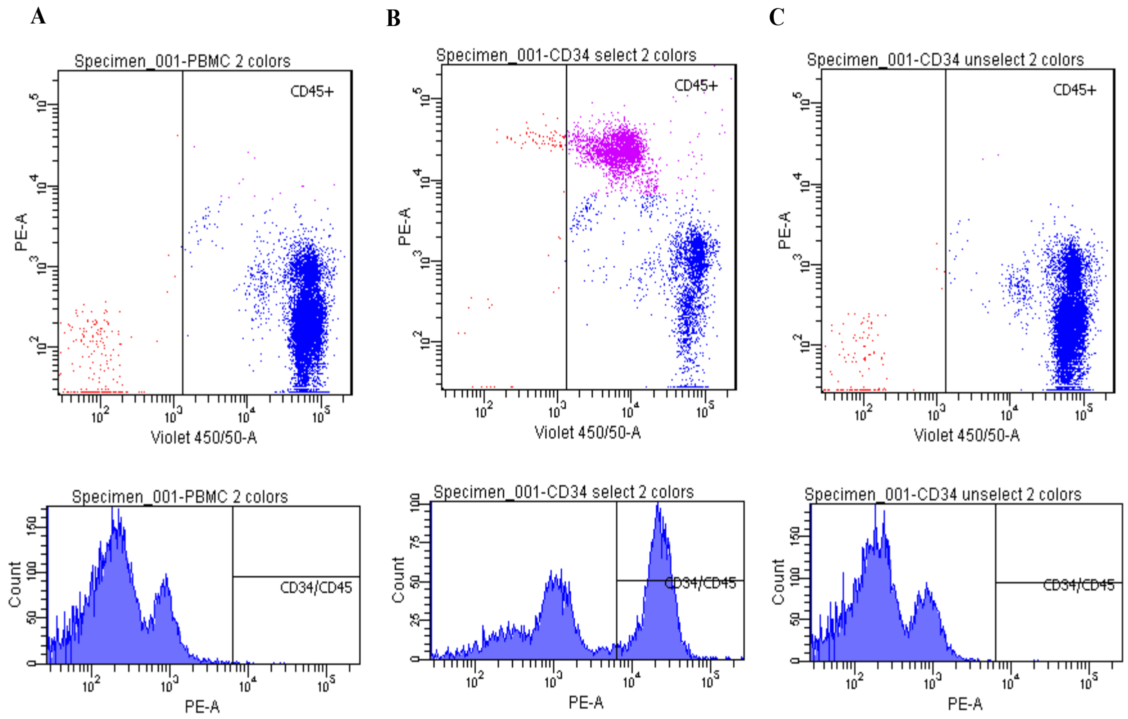
We thank the study volunteers for their time and commitment to this study. Dr. Yan Wang at the UMCP Proteomics Core Facility is thanked for her assistance in the identification of secreted proteins.

Table 3.1. Subject Characteristics

	Endurance Trained (n=14)	Active (n=15)	Inactive (n=12)
Age (yr)	30±1.5	30±1.3	28±1.4
BMI (kg/m^2)	22±0.6	23±0.5	23±0.6
Body Fat (%)	12.3±1.8	13.4±1.5	19.7±1.7*#
Absolute VO$_2$max (mL/min)	3941±264	3599±219	2536±250*#
Relative VO$_2$max (ml/kg/min)	62±2.1	53.1±1.7*	37±2*#
SBP (mm Hg)	115±3.6	120±3	122±3.4
DBP (mm Hg)	70±2	75±1.6*	79±1.8*
MAP (mm Hg)	85±2.2	90±1.8	93±2.1*
Glucose (mg/dl)	81±2.8	83±2.3	83±2.7
Cholesterol (mg/dl)	172±8.6	177±7.2	167±8
HDL-C (mg/dl)	68±5.6	66±4.6	56±5.3
LDL-C (mg/dl)	90±7	96.7±5.8	98.4±6.7
VLDL-C (mg/dl)	14±1.7	14±1.4	13±1.7
TC/HDL	2.6±0.2	2.8±0.2	3.1±0.2
LDL/HDL	1.4±0.2	1.6±0.2	1.9±0.2
Triglycerides (mg/dl)	71±8.8	71±7.3	68±8.3

*Significantly different from Endurance Trained; #Significantly different from Active

Figure 3.1



Total PMBCs

CD34+ selected PBMCs

Unselected CD34- PBMCs

Figure 3.1. Flow activated cell sorting (FACS) plots representing PE-conjugated CD34/V450-conjugated CD45 cells within (A) total PMBCs, (B) CD34+ selected PBMCs using immunomagnetic cell separation procedures and (C) unselected (negative fraction) CD34 cells. Multiple trials have resulted in an isolation purity of 52 ± 3%.

Figure 3.2

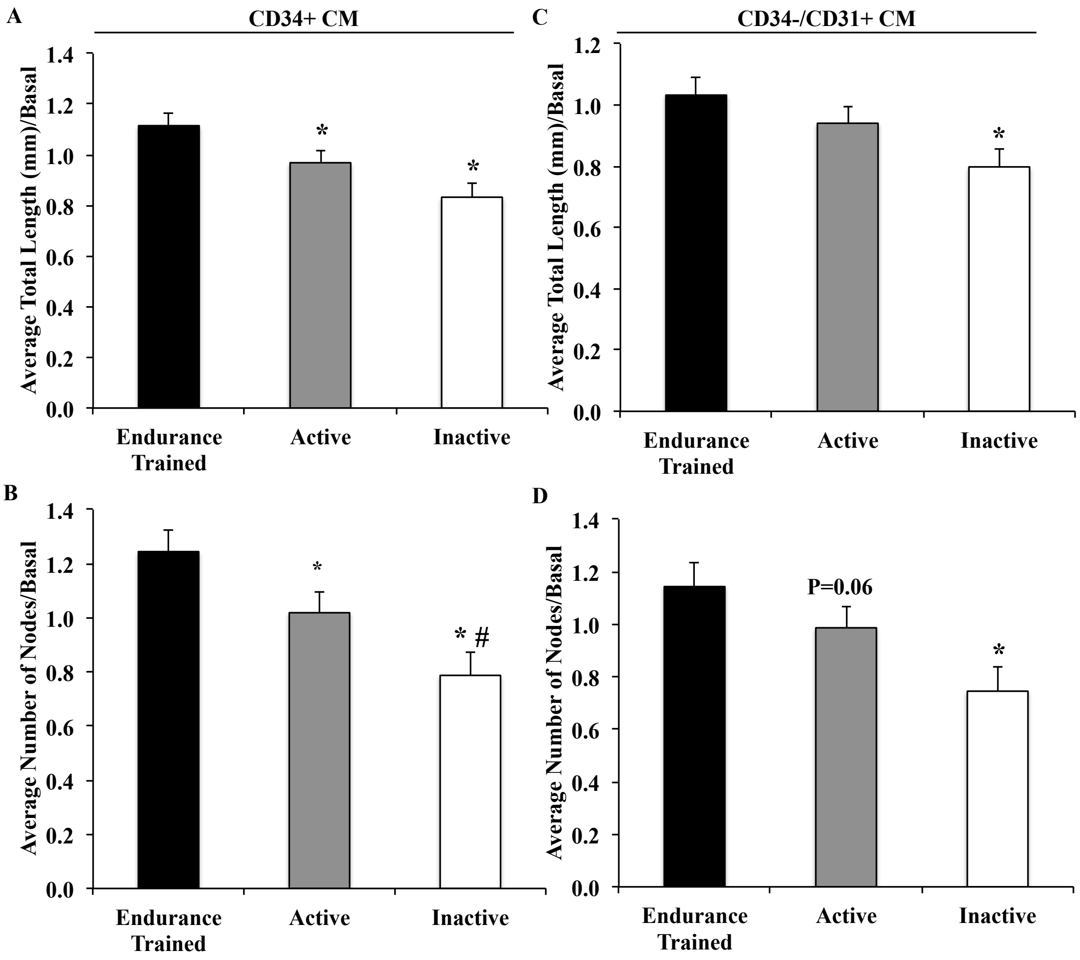


Figure 3.2. Quantification of HUVEC length and complexity after culture with conditioned media from CD34+ (A, B) and CD34-/CD31+ cells (C, D). CD34+ and CD34-/CD31+ CACs were cultured in un-supplemented endothelial growth medium free of growth factors or serum, each at a density of 100,000 cells per well of a fibronectin coated plate. Media was collected after 48hrs of culture from each cell type. Results are presented as each condition normalized to the basal (EBM-2) condition to control for daily variability in HUVEC growth and passage number with each assay. Images were taken under 10x magnification. * Indicates statistically significant difference from high endurance trained subjects, # Indicates statistically significant difference from active subjects. Significance accepted at $P \leq 0.05$.

Figure 3.3

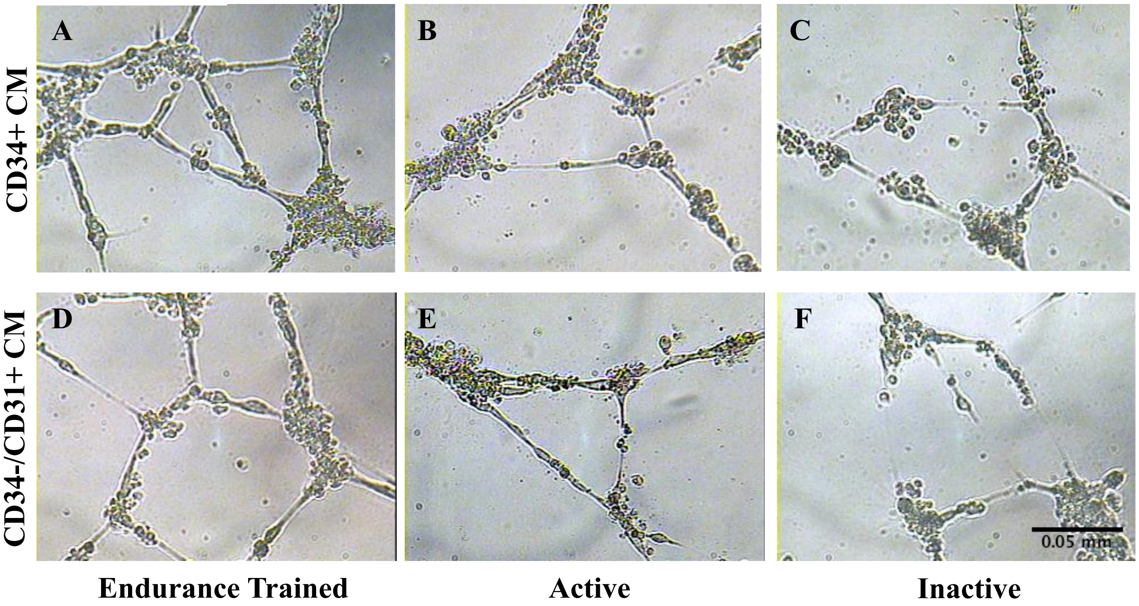

CD34+ CM
A
B
C
CD34-/CD31+ CM
D
E
F
0.05 mm
Endurance Trained
Active
Inactive

Figure 3.3. Representative images of HUVEC cord formation from conditioned media of endurance trained (A, D), active (B, E) and inactive (C, F) circulating angiogenic cells from CD34+ (A, B, C) and CD34-/CD31+ cells (D, E, F).

Figure 3.4

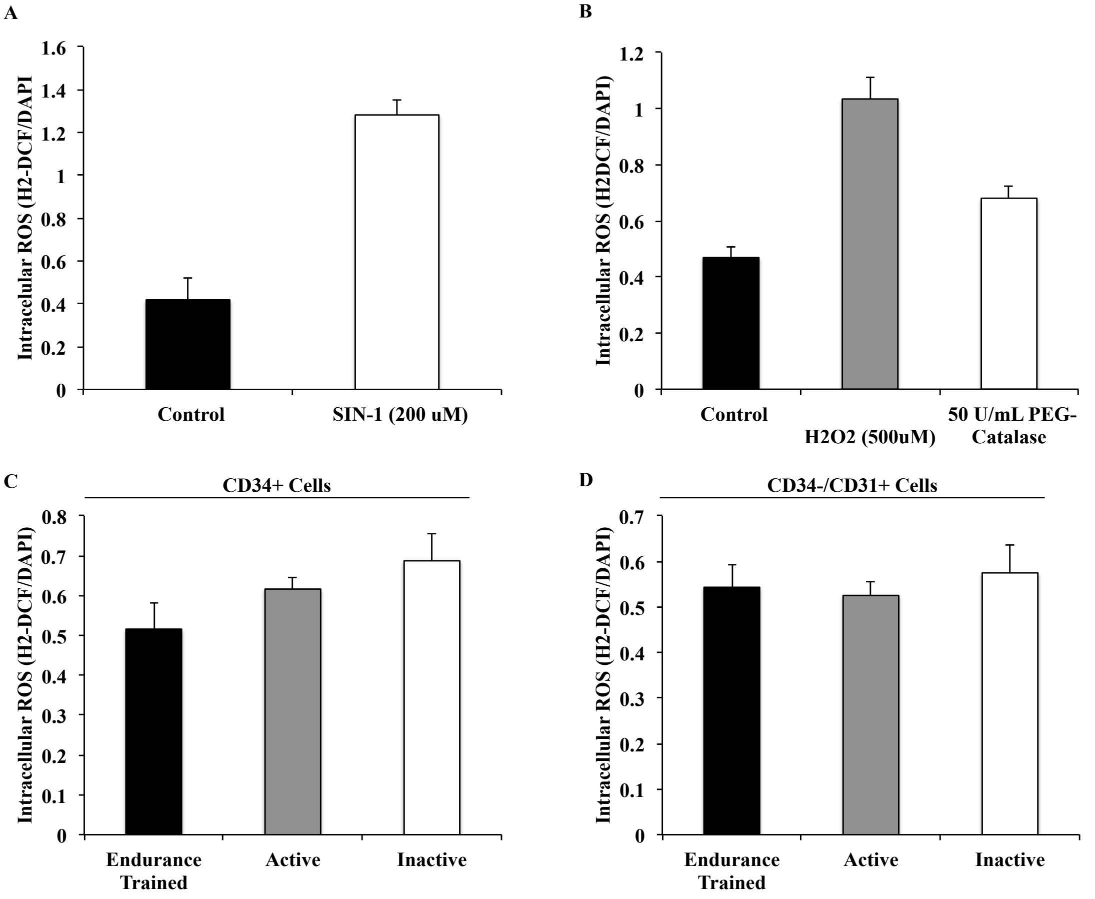


Figure 3.4. Experiments demonstrating the efficacy of the fluorescent probe H2DCF-DA to detect differences in intracellular ROS in CD34+ PBMCs (A-B, n=3 per experiment). In the presence of peroxynitrite donor SIN-1 (200 µM), H2DCF-DA signal increases by more than 200% (A). The H2DCF-DA signal is increased by over 100% in the presence of hydrogen peroxide (500 µM) but pretreatment with PEG-catalase (50 U/mL) reduces this signal by nearly 40% (B). Effects of exercise training status on intracellular reactive oxygen species content in freshly isolated CD34+ (C) and CD34-/CD31+ (D) circulating angiogenic cells (n=12 endurance-trained, n=15 active, and n=12 inactive).

Figure 3.5

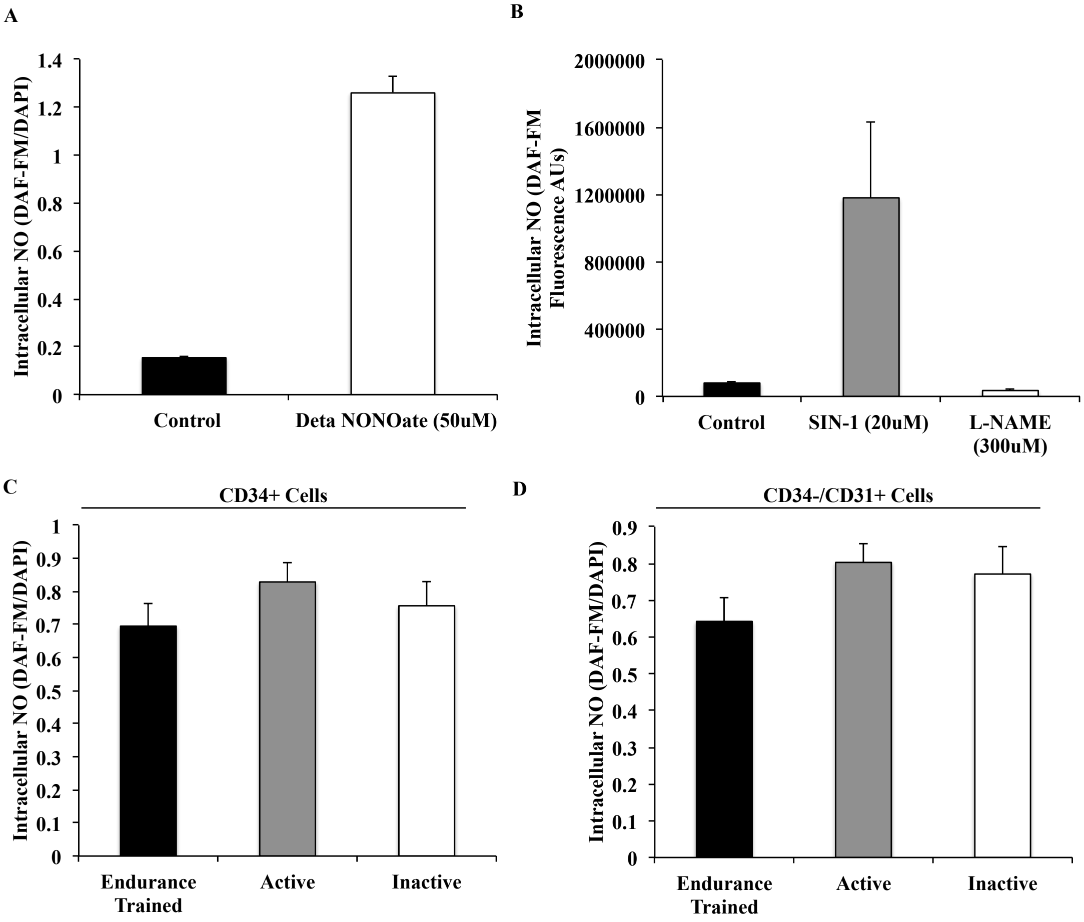


Figure 3.5. Experiments demonstrating the efficacy of the fluorescent probe DAF-FM diacetate to detect differences in intracellular NO in CD34+ and fractioned PBMCs (n=3 per experiment). Greater than 700% increases in DAF-FM signal in the presence of an NO donor, Deta NONOate (50 μM) (A). Over 3-fold increases in DAF-FM signal in the presence of SIN-1 (20 μM), and 56% reduction in DAF-FM signal with L-NAME treatment (300 μM) compared to control (B). Effects of exercise training status on intracellular nitric oxide content in freshly isolated CD34+ (C) and CD34-/CD31+ (D) circulating angiogenic cells (n=12 endurance-trained, n=15 active, and n=12 inactive).

Figure 3.6

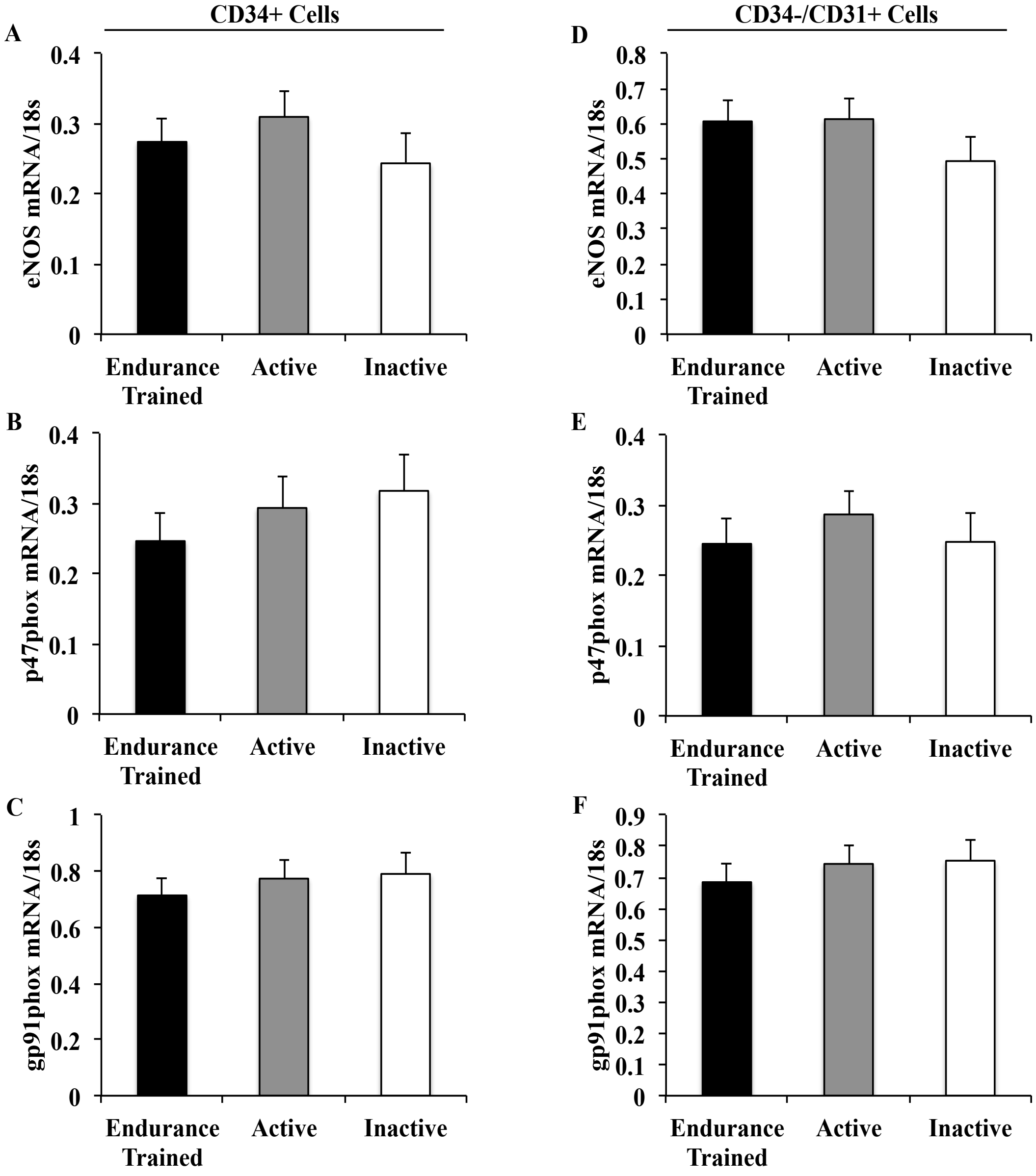


Figure 3.6. Effects of exercise training status on eNOS, p47^{phox}, and gp91^{phox} mRNA expression for freshly isolated CD34+ circulating angiogenic cells (A-C; n= 13 endurance-trained, n=13 active and n=8 inactive), and CD34-/CD31+ circulating angiogenic cells (D-F; n=14 endurance-trained, n=15 active and n=11 inactive).

Figure 3.7

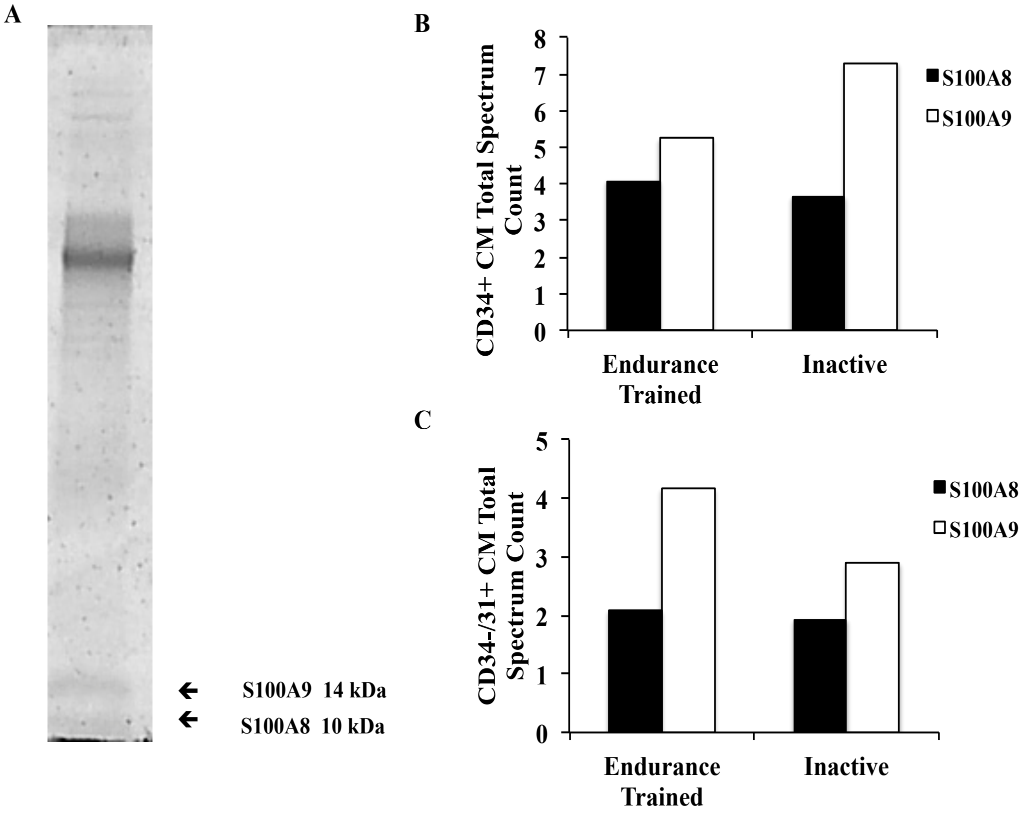


Figure 3.7. Total protein gel of CM from cultured CACs (A) showing bands at 10 kDa (S100A8) and 14 kDa (S100A9). Semi-quantitative analysis of S100A8 and S100A9 in conditioned media of cultured CD34+ cells (B) and CD34-/CD31+ cells (C) using mass spectrometry spectrum counts (n=1) to identify proteins present in conditioned medium.

Figure 3.8

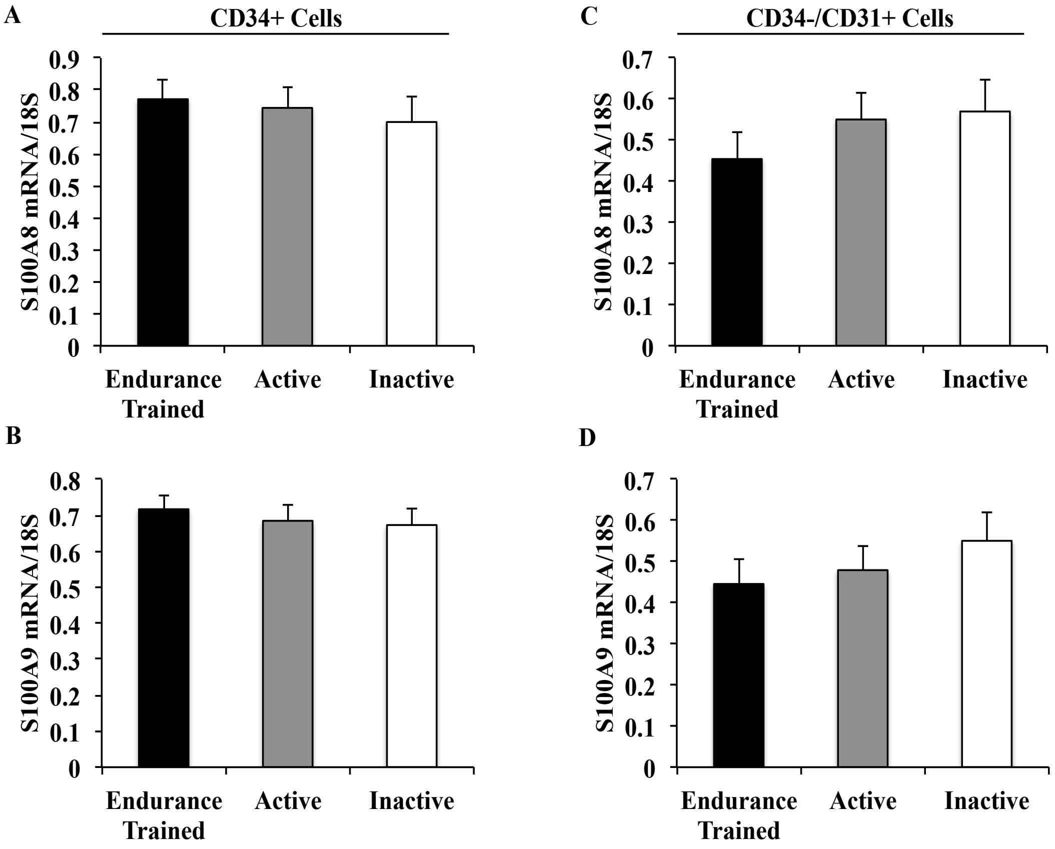


Figure 3.8. Effects of exercise training status on S100A8 and S100A9 mRNA expression for freshly isolated CD34+ circulating angiogenic cells (A-B; n= 13 endurance-trained, n=13 active and n=8 inactive) and CD34-/CD31+ circulating angiogenic cells (C-D; n=14 endurance-trained, n=15 active and n=11 inactive).

Figure 3.9

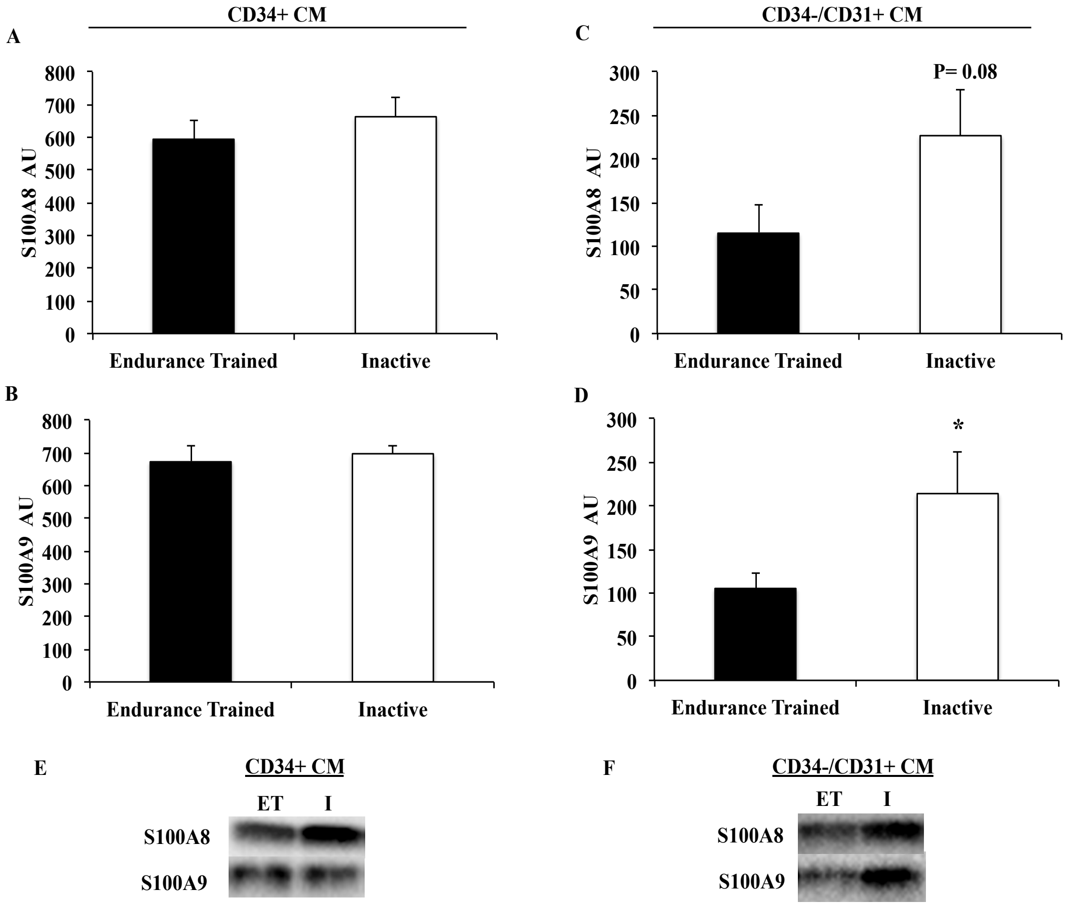


Figure 3.9. Secreted S100A8 and S100A9 protein content in cultured CD34+ (A, B) and CD34-/CD31+ (C, D) cell conditioned media (CM) from endurance-trained and inactive subjects (n=12 per group in CD34+ blots and n=11 per group in CD34-/CD31+ blots). Values were normalized to cell number (100,000 cells/well) as equal volumes of CM were loaded for western blot analysis. Representative blots for CD34+ CM (E) and CD34-/CD31+ CM (F). * Indicates statistically significant from endurance-trained subjects. Significance accepted at $P \leq 0.05$.

Figure 3.10

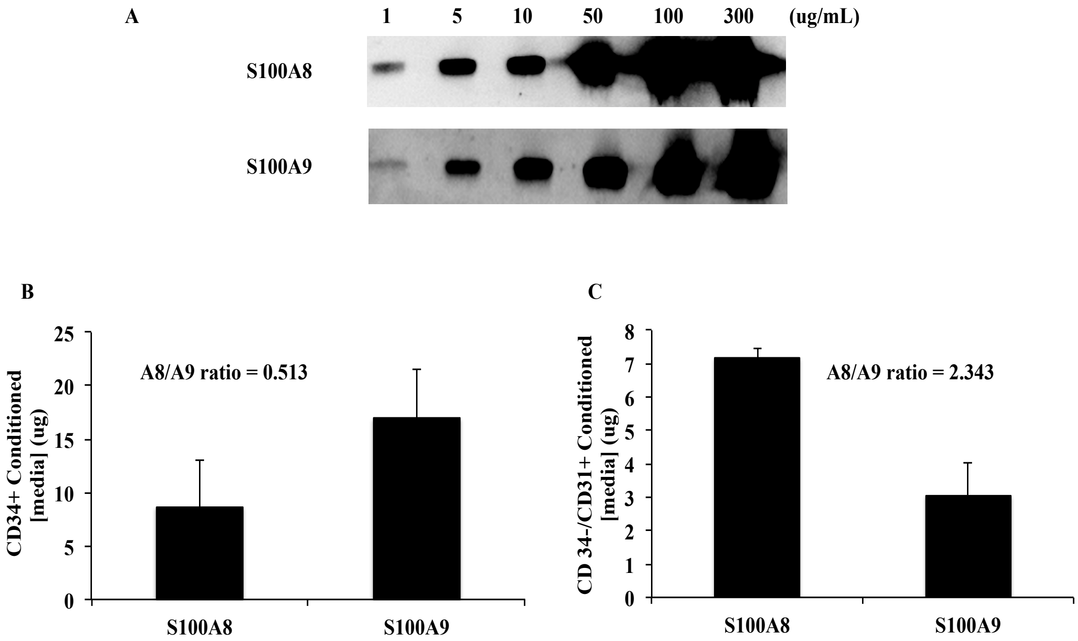
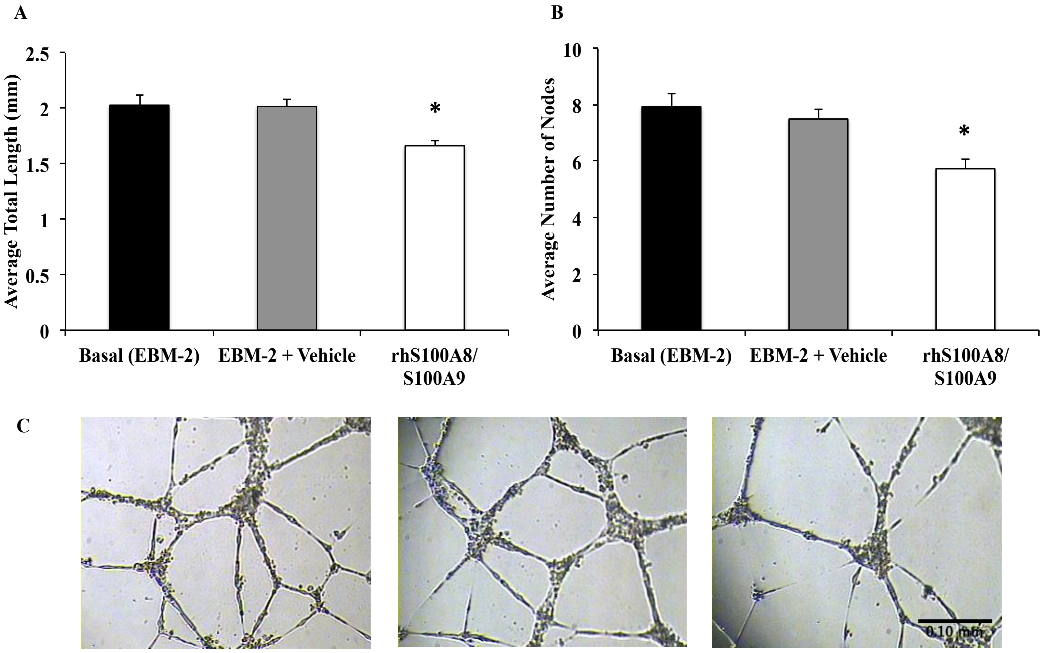


Figure 3.10. Representative western blots depicting the standard curve (1ug/mL-300ug/mL) of recombinant human S100A8/A9 used to estimate the approximate concentrations of each protein in the CD34+ CM and CD34-/CD31+ CM (A). Approximate concentrations of S100A8 and S100A9 present in n=3 CD34+ CM samples (n=2 inactive subject CM and n=1 endurance-trained subjects' CM) (B) and n=3 CD34-/CD31+ CM (n=2 inactive subject CM and n=1 endurance-trained subject's CM) (C) as estimated through western blots using this standard curve.

Figure 3.11



Basal (EBM-2) Condition

EBM-2 + Vehicle

rhS100A8/S100A9

Figure 3.11. Effects of recombinant human S100A8 and S100A9 (7.18 µg/mL and 3.06 µg/mL, respectively for a total volume of 150 µL/well) on HUVEC cord length (A) and complexity (B) compared to basal media and vehicle conditions and representative images from each (C). In these experiments, each condition was assessed in samples collected from six independent cell culture wells from multiple culture plates collected on different days. All experiments were conducted on cells from the same passage number (P4). * Indicates statistically significant from endurance-trained subjects. Significance accepted at $P \leq 0.05$.

Chapter 4: Paracrine actions of NSTEMI patients’ CD34-/CD31+ CACs impair HUVEC tube formation and are mediated by TLR4

The following is a manuscript in preparation based on my final dissertation work.

Paracrine actions of NSTEMI patients' CD34-/CD31+ CACs impair HUVEC tube formation and are mediated by TLR4

Rian Q. Landers-Ramos[1], Ryan M. Sapp[1], Kajal Hamidzadeh[2], Emily VandeWater[1], Jennifer Macko[1], Shawn Robinson[3], David M. Mosser[2], Yan Wang[2], Eva R. Chin[1], Espen E. Spangenburg[1], Steven J. Prior[4], James M. Hagberg[1]

[1]Department of Kinesiology, School of Public Health, University of Maryland, College Park, MD

[2]Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

[3]Baltimore VA Medical Center, Baltimore, MD

[4]University of Maryland School of Medicine and Baltimore VA GRECC, Baltimore, MD

Correspondence: James M. Hagberg, Ph.D., 2134E SPH Bldg, University of Maryland, 255 Valley Dr, College Park, MD 20742-2611. 301-405-2487 (TEL), 301-405-5578 (FAX). hagberg@umd.edu

Running Title: paracrine actions of NSTEMI CACs

ABSTRACT

We sought to determine if conditioned media (CM) from CD34-/CD31+ circulating angiogenic cells (CACs) of non ST-segment elevation myocardial infarction (NSTEMI) patients results in impaired tube formation and whether pre-treatment of human umbilical vein endothelial cells (HUVECs) with inhibitors for receptors of S100A8/A9 improves tube formation to levels similar to those observed when using CM from endurance-trained athletes. Fasting blood was drawn from 10 athletes aged 18-35 yrs and 10 NSTEMI patients aged 50-85 yrs for isolation and culture of CACs. CM from cultured CACs was used in an angiogenesis assay with and without pretreatment with TAK-242, a TLR4 inhibitor, or FPS-ZM1, a RAGE antagonist. CM derived from NSTEMI patient CACs resulted in 9% lower tube length ($P<0.05$) and 18% fewer nodes ($P=0.08$) compared to CM from athletes. Pre-treatment with TAK-242, but not FPS-ZM1, improved tube length ($P<0.05$), number of branches ($P<0.05$) and number of meshes ($P<0.05$) in the NSTEMI patients compared to CM alone but did not affect tube formation in the athletes' CM conditions ($P>0.05$). mRNA expression and S100A8 and S100A9 content in CM was higher in the NSTEMI patients' CACs compared to the athletes ($P<0.05$). Recombinant S100A8/A9 experiments also reduced tube formation and this effect was rescued with TAK-242 treatment ($P<0.05$). Flow cytometry analyses suggest that CACs from NSTEMI patients contain fewer T-cell and monocyte markers compared to athletes (n.s.). These results suggest that S100A8 and S100A9 present in CM from CACs of NSTEMI patients impair HUVEC tube formation and that these effects appear to be mediated through TLR4.

INTRODUCTION

Circulating angiogenic cells (CACs) are involved in the repair and maintenance of the vascular endothelium. Originally believed to incorporate directly into the endothelium (4), it is now generally accepted that CACs function mainly through paracrine mechanisms (79, 175). A study recently published by our lab found that conditioned medium (CM) from CD34-/CD31+ CACs of physically inactive but otherwise healthy, younger adults impaired human umbilical vein endothelial cell (HUVEC) tube formation compared to their endurance exercise trained or recreationally active counterparts (88). We identified higher S100A8 and S100A9 content in the CM generated from CACs from inactive individuals' compared to endurance-trained subjects, and found that these proteins were, at least partially, mediating the observed differences in tube formation (88). S100A8 and S100A8 are members of the S100 family of calcium binding proteins that are well known for their roles in inflammatory diseases (5, 17, 18, 153). They can function independently as monomers or as an S100A8/A9 heterodimer complex (153). High plasma levels of these proteins have been recently identified as risk factors for cardiovascular disease (CVD) (5, 17) with plasma levels predicting future CV events in middle-aged healthy individuals (17, 18). However, the direct effects of S100A8 and S100A9 secreted by CACs on endothelial cells are less understood.

CD31 (aka PECAM-1) is a cell surface marker found on circulating endothelial cells, neutrophils, monocytes, haematopoietic progenitor cells, T cells, and B cells as well as other subsets of cells (76). CD31+ cells have known roles in angiogenesis and CD34-/CD31+ CACs have comparable, if not greater, angiogenic potential than the more commonly studied progenitor cell lines (78). In addition to their higher frequency in peripheral blood, previous work from our lab indicates that CD34-/CD31+ CACs are potentially more susceptible to modulation by lifestyle factors than CD34+ CACs (64, 88).

It is recognized that the CACs from CVD patients are lower in number and have diminished function compared to those from their healthy counterparts (76). Indeed, Kim et al. (2014) recently demonstrated that CD31+ CACs from coronary artery disease (CAD) patients exhibited lower cell migration rates, lower adhesion, and lower tube formation when co-cultured with HUVECs, and showed greater apoptosis compared to the CD31+ CACs from healthy controls (76). Additionally, this study found that the CD31+ CACs from CAD patients exhibited lower levels of angiogenic T-cell surface markers and higher levels of monocyte/macrophage markers (76) as well as an upregulation of inflammatory genes and downregulation of angiogenic genes compared to healthy subjects. This study highlights the importance of considering the functional capacity of CACs to be used for autologous cell therapy.

Additionally, although other functional traits of CD31+ CACs have been studied in CVD populations (76), differential secretion of soluble factors, specifically S100A8 and S100A9, have not yet been explored in patient populations. The purpose of this study is to determine if CM derived from CD34-/CD31+ CACs of non ST-segment elevation myocardial infarction (NSTEMI) patients results in impaired tube formation due to higher levels of S100A8 and S100A9 when compared to a healthy endurance-exercise trained population. The major receptors for S100A8 and S100A9 are toll-like receptor 4 (TLR4) (155) and the receptor for advanced glycation end products (RAGE) (11), both of which are present on HUVECs and have been found to activate downstream inflammatory pathways upon S100A8 and S100A9 binding (26, 155, 162). Thus, we aimed to test whether pre-treatment of HUVECs with inhibitors for these major receptors of S100A8/A9 will improve tube formation to levels similar to those observed when using CM from endurance-trained athletes. Finally, we sought to characterize other surface markers present on CD34-/CD31+ CACs from each group to help explain potential differences in functional properties between NSTEMI patients' and healthy endurance-trained subjects' CACs.

METHODS

Ethical Approval

The University of Maryland College Park Institutional Review Board approved all study procedures and subjects provided written informed consent. The study procedures conformed to the standards set by the Declaration of Helsinki.

Screening

Potential NSTEMI patients were recruited from patients admitted to the Baltimore Veterans Affairs Medical Center. The patients participating in this study were men 50-88 yrs of age with a BMI between 18-40 kg/m^2, a history of or current symptomatic CAD and a recent uncomplicated myocardial infarction without ST segment elevation. Participants were not excluded based on race/ethnicity or based on medications due to their necessity in this population. A list of current medications taken by our patient population can be found in Table 4.1. In this study, endurance-trained athletes were used as a model of optimal CAC function for comparison to the NSTEMI group. Endurance-trained subjects were healthy, nonsmoking, men aged 18-39 yrs with no history of CV or metabolic disease. Potential subjects were initially screened by telephone or email, and reported to the laboratory following an overnight fast for a screening visit to verify eligibility. Subjects were categorized based on their reported physical activity over the last 5 years and confirmed via maximal oxygen consumption (VO_2max) testing after achieving superior values according to the American College of Sports Medicine (ACSM) (107). Specifically, the endurance-trained group (n=13) reported performing >4 hours/week of moderate to high intensity endurance exercise. Exclusion criteria for the athletes were as follows: systolic blood pressure ≥130 mm Hg, diastolic blood pressure

≥90 mm Hg, serum total cholesterol ≥200 mg/dl; low-density lipoprotein-cholesterol ≥130 mg/dl; high-density lipoprotein-cholesterol ≤ 35 mg/dl; fasting glucose ≥100 mg/dl.

Maximal Graded Exercise Test, Body Composition and Blood Sampling

For the endurance-trained group, a screening blood sample was obtained for assessment of fasting serum triglyceride (TG), lipoprotein lipids, and glucose (Quest Diagnostics, Baltimore, MD). Height, weight, seated blood pressure and BMI were measured, and body composition was assessed using the 7-site skinfold procedure (62). VO_2max was assessed using a constant-speed treadmill protocol with 2–3% increases in incline every 2 min until exhaustion. The treadmill speed was based on each subject's experience, typical run speed, and heart rate, such that VO_2max was achieved within 6–12 min. Pulmonary ventilation and expired gas concentrations were analyzed in real time using an automated computerized indirect calorimetry system (Oxycon Pro, Viasys). VO_2 was considered maximum if a plateau was achieved (increase in VO_2 of < 250 ml/min with increased work rate). In the absence of a clear plateau, tests had to meet at least two of the following secondary criteria: a respiratory exchange ratio > 1.10, a rating of perceived exertion >18, and a peak heart rate within 10 beats/min of the age-predicted maximum.

On the testing day of blood sampling for CACs, the endurance-trained subjects reported to the laboratory in the morning after an overnight (~12 hr) fast. Subjects performed their normal exercise routine 16-24 hours prior to the blood sampling. A sample of 50mL of blood was drawn using EDTA-tubes (Becton Dickinson) for isolation of CD34-/CD31+ CACs and plasma. Blood sampling for the NSTEMI group was performed at the Baltimore Veterans Affairs Medical Center. Briefly, 50 mL blood samples were obtained in EDTA-tubes between 24-72 hours after presentation to the emergency room, prior to cardiac catheterization procedures. Patients were NPO overnight prior to blood sampling. Samples were then immediately transported to the University of Maryland, College Park for processing.

Immunomagnetic Cell Separation

Peripheral blood mononuclear cells (PBMCs) were isolated from the venous blood samples using density gradient centrifugation (Ficoll, GE Healthcare). The CD34+ fraction was purified using multiple rounds of immunomagnetic cell separation according to the manufacturer's instructions (EasySep® Immunomagnetic Cell Separation Kits, STEMCELL Technologies) using an antibody (Ab) specific for CD34. CD31+ cells were selected from the CD34- fraction of cells and purified as described above using and Ab specific for CD31 (hereby referred to as CD34-/CD31+). This isolation approach has been published previously by our lab (63, 64, 87) and the purity of our isolation is equivalent to or greater than other published results also using non-mobilized blood (3, 6, 41, 128, 160).

CAC Culture and Conditioned Media

CD34-/CD31+ CACs were re-suspended in un-supplemented endothelial growth medium free of growth factors or serum, (EBM-2, Lonza) with 1% Antibiotic-Antimycotic (Invitrogen) each at a density of 300,000 cells per well as determined by hemocytometer counts. Cultures were maintained for 48 hrs in a humidified incubator at $37^{o}C$ and 5% CO_2. After incubation, the CM from all wells was withdrawn and combined into one tube and clarified by spinning at 2,500xg for 20 min to remove cells and debris from the medium. CM not used for immediate experiments was flash frozen in small aliquots and stored at -80^{o}C until further analyses.

Angiogenesis Assay

The angiogenesis tube formation assay (2, 19, 39, 125) was performed as done previously (87). Briefly, culture plates were coated with Reduced-Growth Factor Matrigel (BD Biosciences) and the matrigel was left to solidify for 30 min at 37^{o}C and 5% CO_2. Each condition was performed in duplicate. Each well contained 20,000 HUVECs and either endothelial basal medium (EBM-2) free of growth factors and serum or CM from CD34-/CD31+ CACs. For some conditions, HUVECs were pretreated with 500nM TAK-242, a TLR4 inhibitor, 1μM FPS-ZM1, a RAGE antagonist, or both for 10 minutes before exposure to CM. Concentrations of each drug treatment were determined through manufacturer recommendations as well as previous studies employing these drugs for treatment of HUVECs, and then further optimized in our laboratory. Additional wells were prepared with a similar amount of fresh EBM-2 as a positive control and DMSO vehicle controls. The average HUVEC passage used in the angiogenesis assay for endurance-trained and NSTEMI subjects were 4.1±0.4 and 4.2±0.3 respectively (n.s.). These plates were cultured for 16 hrs at 37^{o}C and 5% CO_2. The cultures were then visualized under a light microscope and 5 images were photographed from the center and periphery of each well. These images were then coded and assessed for HUVEC tube length, number of nodes, meshes and branches using the ImageJ Angiogenesis Analyzer software program. This program defines nodes as a connecting point between two branches, meshes as completely enclosed areas created by tube segments, and branches as tube segments sprouting from nodes but not forming complete meshes. Results are presented as each condition normalized to the EBM-2 condition to control for daily variability in HUVEC growth and passage number within each assay.

Mass Spectrometry, Western Blot Analysis and S100A8 and S100A9 Expression

Proteomics techniques were used to determine proteins present in the CD34-/CD31+ CM from the endurance-trained and NSTEMI groups. Spectral counts for n=3 per group were used as an initial non-labeled method providing semi-quantitative data (99). Subsequently, these findings were quantitatively confirmed using immunoblotting analyses on n=10 per group. Media was concentrated using Amicon Ultra 0.5 3kD centrifugal filter devices (Millipore). The soluble proteins in the supernatant were subjected to digestion with trypsin Lys-C. Samples were analyzed using a ThermoFinnigan LTQ Orbitrap XL mass spectrometer and matched to the UniProtKB database. Western blot analyses were used to assess differences in two identified S100 proteins with specific antibodies for S100A8 (R&D Systems) and S100A9 (Santa Cruz Biotech) as done previously (87). Membranes were washed and then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Cell Signaling; 1:1000) or HRP-conjugated anti-goat IgG (Novus; 1:5000) secondary antibodies. Blots were developed using Super Signal ECL reagents (Thermo Scientific) and bands were visualized and quantified using Chemi-Doc Imaging System and Software (BioRad). Values were normalized to the 300,000 cells/well used to generate the CM.

Assessment of Gene Expression by RT-PCR

Total RNA was extracted from freshly isolated CACs using the TriZol reagent, quantified using a spectrophotometer (BioTek H1 Synergy Hybrid Reader, Winooski, VT) and reverse transcribed to cDNA (Life Technologies, Grand Island, NY).

Quantitative real-time polymerase chain reaction (PCR) was performed using Applied BioSystems 7300 Real-Time PCR System. Primer Assays were purchased from IDT (Coralville, IA) and optimal concentration for efficacy of >90% was determined. Primer sequences are listed in Table 4.2. Each reaction was performed in duplicate on a 96-well plate and contained iTaq Universal Probes Supermix (Biorad, Hercules, CA), respective primer probe, and the cDNA template. The PCR conditions used were as follows: 95°C for 3 min, followed by 50 cycles of 95°C for 15 sec, and 60°C for 45 sec. mRNA expression values are presented as $2^{-\Delta CT}$ where ΔC_T is the cycle threshold (C_T) of the target gene minus *GAPDH* control for each condition. *GAPDH* primers were used as a control gene and *GAPDH* cycle thresholds (CTs) were not different across time in the present study.

Recombinant S100A8 and S100A9 HUVEC treatment

To confirm the direct effects of S100A8 and S100A9 on HUVEC tube formation, the concentrations and proportions estimated in the CM of the athletes' and NSTEMIs' CD34-/CD31+ CACs were used in an angiogenesis assay. Using recombinant human S100A8/A9 (Life Technologies), a standard curve was established using immunoblotting to allow us to estimate the concentration of each protein that the HUVEC cells were exposed to in a subset of CM samples from each group (n=3/group). Based on these estimations, 2.9 μg/mL of recombinant human S100A8 (ProSpec Bio) and 0.8 μg/mL of recombinant human S100A9 (Life Technologies) were used to simulate the athlete conditions and 6.7 μg/mL of recombinant human S100A8 and 1.4 μg/mL of recombinant human S100A9 were used to match the NSTEMI conditions. These proteins were added to a HUVEC-based tube formation assay and compared to the positive control prepared with EBM-2 and a vehicle control.

In order to confirm the role of TLR4 in the inhibitory actions of these proteins on HUVECs, a condition in which the HUVECs were pretreated with 500nM TAK-242 before exposure to NSTEMI concentrations of S100A8 and S100A9 was also included. In these experiments, each condition was assessed in samples collected from n=6 independent cell culture wells from multiple culture plates collected on different days to confirm replication of our findings. All experiments were conducted on cells from the same passage number (P4). As described above, each well contained 20,000 HUVECs. The plates were cultured for 16 hrs at 37^oC and 5% CO_2. The cultures were then visualized under a light microscope and 5 random images were photographed per well.

Signaling Experiments

HUVECs were harvested and 180,000 cells/condition were used for experiments. HUVECs were exposed to either endothelial basal medium free of growth factors and serum or CM from CD34-/CD31+ CACs for 1 hour at 37^oC and 5% CO_2. For some conditions, HUVECs were pretreated with 500nM TAK-242, a TLR4 inhibitor, 1µM FPS-ZM1, a RAGE antagonist, or both for 10 minutes before exposure to CM. HUVECs were then spun at 1,000 x g for 5 min, supernatant was poured off and 1x Mueller buffer containing protease inhibitors was used to collect and lyse the cells. Cells were incubated at 4^oC for 30 min in Mueller buffer while rotating, then spun down and the homogenate was collected in aliquots and stored at -80^oC for future experiments. The average HUVEC passage used in signaling experiments for endurance-trained and NSTEMI subjects was 4.1±0.4 and 4.2±0.3 respectively (n.s.). For immunoblot analyses, samples (n=6/group) were thawed and protein concentration was determined using a BCA protein assay (Thermo Scientific). Western blot analyses were used to assess differences in selected proteins involved in downstream signaling of TLR4 and RAGE using specific antibodies for p38 (phospho-p38 Thr180/Tyr182, 1:1000; total p38, 1:1000), ERK1/2 (phospho-ERK1/2 Thr202/Tyr204, 1:2000; total ERK1/2, 1:1000), NFκBp65 (phospho-NFκBp65, 1:1000; total NFκBp65, 1:1000) and IκBα (1:1000). All antibodies were purchased from Cell Signaling. Membranes were washed and then incubated with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG secondary antibodies (Cell Signaling; 1:1000). Blots were developed using Super Signal ECL reagents (Thermo Scientific) and bands were visualized and quantified using Chemi-Doc Imaging System and software (BioRad). The average basal condition from each membrane served as a blot control and values are presented as phospho/total target protein after normalizing to total protein, or normalized to total protein when no phosphorylation event was assessed.

S100A8/A9 ELISA

Plasma calprotectin (S100A8/A9 heterodimer complex) levels were measured using an enzyme-linked immunosorbant assay (ELISA) Kit (BMA Biomedical). This kit is designed to detect the heterodimer complex using a capture antibody that recognizes an epitope present on the heterodimer complex but that is not present on either of the monomers. The average intra-assay coefficient of variation was 6.9% and all samples were assayed on the same plate to avoid inter-assay variability. This assay has a sensitivity of ~1 ng/mL.

Fluorescent Activated Cell Sorting

In an effort to further characterize these cells, flow cytometry was used to determine the percent of selected CD34-/31+ CACs that were also positive for the T-cell surface marker, CD3, macrophage surface marker, CD11b, and the monocyte surface marker, CD14. CD34-/31+ cells were isolated from total PBMCs using immunomagnetic isolation methods as described above. A total of $1x10^6$ cells were FcR blocked (Miltenyi Biotech), immunostained with anti-human CD3-APC (BD Biosciences), CD11b-Pacific Blue (BD Biosciences), or CD14-PerCP (BD Biosciences), and fixed in 2% paraformaldehyde. Flow cytometry analyses were performed in the University of Maryland Flow Cytometry Core Facility with a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (FlowJo, LLC) using the appropriate isotype controls (BD Biosciences).

Statistics

Statistical analyses were completed using IBM SPSS Statistics 21. Assumptions of homoscedasticity and normality were verified for all outcome measures. Tube formation and HUVEC signaling data were analyzed using multivariate ANOVA with pairwise comparisons where appropriate. t-tests were used for western blot, ELISA and qPCR analyses. Statistical significance was accepted at $P \leq 0.05$. Values are expressed as mean ± standard error of the mean.

RESULTS

Subject characteristics

Subject characteristics can be found in Table 4.3. VO_2max assessments confirmed that the endurance-trained athletes were, by design, substantially above average in aerobic fitness for their age group according to American College of Sports Medicine Guidelines (107). Despite having suffered a recent cardiac event, NSTEMI patients were relatively lean with well-controlled blood pressure and plasma lipoprotein-lipid profiles.

Angiogenic tube formation with conditioned media

CM derived from NSTEMI patient CACs resulted in 9% lower total tube length (P<0.05; Figure 4.1A) and 18% fewer nodes (P=0.08; Figure 4.1B) compared to CM from endurance-trained athletes. Although the NSTEMI patients had a lower number of tube meshes and branches (Figure 4.1C and 4.1D), neither of these differences were significant compared to the endurance-trained athletes (P>0.05). Pre-treatment with TAK-242 significantly improved total tube length (P<0.05), number of branches (P<0.05), and number of meshes (P<0.05) in the NSTEMI patients compared to CM alone (Figure 4.1A-C). However, neither FPS-ZM1 treatment nor combined TAK-242 + FPS-ZM1 treatments significantly altered tube formation in the NSTEMI patients (P>0.05). There were significantly fewer branches in the NSTEMI group compared to the athletes when HUVECs were pretreated with FPS-ZM1 (P<0.05; Figure 4.1D). Drug treatments did not affect tube formation in the endurance-trained cell CM conditions (P>0.05). Representative images from each condition can be found in Figure 4.2.

Detection of Proteins in Conditioned Media

In an attempt to compare secreted paracrine factors from CACs of athletes vs. NSTEMIs, CM from CD34-/CD31+ cells from each group were analyzed by mass spectrometry. A full list of identified proteins can be found in Supplemental table 4.I. As found previously, two targets from the S100 family of proteins, S100A8 and S100A9, were identified in the secretome of CD34-/CD31+ cells of both groups; with spectrum counts from S100A8 and S100A9 being 78% and 76% greater, respectively, in NSTEMIs compared to athletes (Figure 4.3A-B). Follow up immunoblotting (n=10/group) confirmed that there was 165% more S100A8 content in the CM from the NSTEMI group compared to the athletes ($P<0.05$; Figure 4.3C) and 153% greater S100A9 content in CD34-/CD31+ CM of NSTEMI subjects compared to endurance-trained athletes ($P<0.05$; Figure 4.3D).

S100A8 and S100A9 Gene Expression

qPCR analyses of CD34-/CD31+ CACs indicated that there was significantly greater expression of both S100A8 and S100A9 in the CACs of NSTEMIs compared to athletes ($P<0.05$ for both; Figure 4.4A-B).

Recombinant human S100A8 and S100A8 treatment

Recombinant human S100A8 (rhS100A8) and S100A9 (rhS100A9) were added to a HUVEC-based tube formation assay in the concentrations and proportions that were estimated to be present in the CD34-/CD31+ CM of endurance-trained athletes and NSTEMI patients. There were no significant differences observed between the basal condition and the condition using the concentrations of rhS100A8 and rhS100A9 estimated to be present in the athlete CM. However, when using concentrations of each protein estimated to be present in the NSTEMI group CM, there was a 23% reduction in tube length (Figure 4.5A; $P<0.05$), a 41% reduction in number of nodes (Figure 4.5B; $P<0.05$) and a 60% reduction in number of meshes (Figure 4.5C; $P<0.05$) compared to the basal condition. Importantly, this effect was rescued when the HUVECs were pretreated with TAK-242 before exposure to the recombinant proteins such that tube length, number of nodes, and number of meshes were significantly higher than basal and athlete conditions (Figure 4.5A-C; $P<0.05$). Number of tube branches was unchanged with any treatment (Figure 4.5D; $P>0.05$). Interestingly, only TAK-242 treatments also significantly improved tube length and number of nodes compared to basal and athlete conditions (data not shown; $P<0.05$).

Assessment of Signaling Pathways

No significant differences in phosphorylation of p38, ERK1/2 and NFκB p65 or degradation of IκBα were found between groups after a 1-hour exposure of HUVECs to CD34-/CD31+ CM ($P<0.05$; Figure 4.6). HUVEC pretreatment with TAK-242 prior to exposure to the CM from athletes resulted in higher phosphorylation levels of ERK1/2 ($P<0.05$), p38 ($P=0.06$), and NFκB p65 ($P<0.05$) compared to the CM from NSTEMIs (Figure 4.6A-C). Additionally, within the athlete CM group, p- ERK1/2 was significantly higher with the TAK-242 treatment compared to all other conditions ($P<0.05$ for each; Figure 4.6A) and p-NFκB p65 was significantly higher with the TAK-242 treatment compared to basal, FPS-ZM1 and TAK242+FPS-ZM1 conditions ($P<0.05$ for each; Figure 4.6C). However, within the NSTEMI CM group, TAK-242 + FPS-ZM1 treatment combined lead to significantly higher p-ERK1/2 compared to basal and CM alone conditions ($P<0.05$ for both; Figure 4.6A), whereas this treatment resulted in lower p-NFκB p65 compared to CM alone or FPS-ZM1 conditions ($P<0.05$ for both; Figure

4.6C). Additionally, within the NSTEMI CM group, TAK-242 treatment lead to significantly lower p-p38 compared to the basal condition ($P<0.05$; Figure 4.6B). There were no significant effects of group or treatment condition on IκBα ($P>0.05$; Figure 4.6D).

Plasma S100A8/A9

No significant differences in plasma S100A8/A9 heterodimer complex were found between athletes and NSTEMIs ($P>0.05$; Figure 4.7).

Characterization of CD34-/CD31+ CACs

In an attempt to further characterize the selected CD34-/CD31+ CACs in athletes vs. NSTEMIs, we employed FACS analyses to identify percent of selected cells also positive for markers of angiogenic T-cells (CD3), macrophages (CD11b) and monocytes (CD14). Analyses of n=3/group indicates that in athletes, 42% more CD34-/CD31+ CACs were positive for CD3 and 56% more were positive for CD14 compared to NSTEMIs (n.s; Figure 4.8A-B). CD11b markers on CD34-/CD31+ cells were similar between groups (n.s.; Figure 4.8A-B). We followed up on these findings by looking at expression of angiogenic and inflammatory genes in CD34-/CD31+ CACs. There were no significant differences between groups in the angiogenic genes, angiopoietin-1, or FGF-2 ($P>0.05$ for both; Figure 4.9A-B). qPCR analyses of inflammatory genes indicated no significant differences between groups in TNF-α expression ($P>0.05$; Figure 4.9C). However, expression of IL-1α was significantly higher in athletes compared to NSTEMIs ($P<0.05$; Figure 4.9D)

DISCUSSION

It is generally accepted that CVD patients have fewer and lower functioning CACs compared to healthy adults (35, 76). Kim et al. (2014) recently demonstrated that selected CD31+ CACs from CAD patients exhibit lower functional properties compared to their healthy counterparts including adhesion, migration, and tube formation when co-cultured with HUVECs (76). CD34-/31+ CACs function mainly through paracrine mechanisms to aid in the maintenance and repair of the endothelium, but how this function differs between CVD patient populations and optimally functioning healthy individuals is unknown. In this study we demonstrate for the first time that CM derived from CD34-/CD31+ CACs of NSTEMI patients impairs HUVEC tube formation compared to the CM from endurance exercise-trained individuals' CACs. Our data provide further support for paracrine actions of CD34-/CD31+ CACs altering angiogenesis and how CVD might affect these actions.

Previous work from our lab using similar methods to investigate the paracrine effects of CD34-/CD31+ CACs revealed a differential paracrine role of CD34-/CD31+ CACs on HUVEC-based tube formation as a function of chronic physical activity habits in otherwise healthy young individuals (87). We identified higher levels of S100A8 and S100A9 in the CM of inactive individuals compared to endurance-trained individuals and confirmed these proteins to be depressors of HUVEC tube formation (87). As a follow up to these findings, in the current study we sought to determine if S100A8 and S100A9 were the major contributors to the observed lower tube formation when CD34-/CD31+ CM from NSTEMIs was used. Using mass spectrometry analyses on the CM from n=3 from each group, we confirmed that spectrum counts for S100A8 and S100A9 were higher in the NSTEMIs compared to the athletes. These findings were then supported through western blot analyses, which confirmed substantially greater levels of S100A8 and S100A9 in the CM of the NSTEMIs compared to the athletes. Additionally, qPCR analyses confirmed that S100A8 and S100A9 mRNA expression was also substantially higher in the CD34-/CD31+ CACs of NSTEMIs compared to athletes. Collectively, these data indicate that the presence of CVD affects the expression and secretion of S100A8 and S100A9 and provide additional support to our earlier study (87) suggesting that these proteins secreted by CD34-/CD31+ are playing a major role in the regulation of angiogenesis.

In order to empirically test the effects of these proteins on HUVEC tube formation, we employed similar techniques as we had done previously (88) to estimate the concentrations of S100A8 and S100A9 that were present in the CD34-/CD31+ CM from NSTEMIs and athletes, which were then used in an angiogenesis assay. Concentrations of recombinant human S100A8 and S100A9 estimated to be present in the athletes' CD34-/CD31+ CM did not significantly alter tube formation compared to the positive control. However, concentrations of S100A8 and S100A9 estimated to be present in the CM from NSTEMI patients' CD34-/CD31+ CACs resulted in a ~23% lower tube length, ~41% fewer nodes, and ~60% fewer tube meshes compared to the positive control and the athlete conditions. These results support our hypothesis that S100A8 and S100A9 concentrations secreted by the CD34-/CD31+ CACs of the NSTEMIs contribute to the impaired angiogenic actions of these cells. Although S100A8/A9 plasma levels have been identified as risk factors for CVD (17, 43), this is the first study, to our knowledge, to identify S100A8 and S100A9 secreted by NSTEMI patients' CD34-/CD31+ CACs as a negative regulator of angiogenesis.

Interestingly, isolating the effects of just S100A8 and S100A9 on HUVEC tube formation resulted in substantially larger reductions in tube formation than those observed when CM was used. It should be noted that proteomics analyses identified over 250 proteins in the CM from the CD34-/CD31+ CACs. As this specific CAC subtype has known roles in angiogenesis, it is likely that other proteins present interacted with S100A8 and S100A9 to blunt their inhibitory actions or functioned separately to promote tube formation leading to an overall lesser observed effect than when the isolated effects of S100A8 and S100A9 are assessed. Thus, it is important that future studies explore potential interactions between S100A8/A9 and other secreted proteins to identify factors that may serve as endogenous inhibitors of these inflammatory proteins.

To date, two major receptors for S100A8 and S100A9, RAGE and TLR4, have been identified on endothelial cells (26, 153, 155, 162). In this study, we demonstrate that inhibiting TLR4 signaling, but not RAGE, attenuated the negative actions of the NSTEMI patient's CM on HUVEC tube formation. These results suggest that the major factor(s) present in the CM of NSTEMI patients' CACs are acting primarily through TLR4. As TLR4 is a potential receptor for multiple proteins present in our CM, we sought to empirically test whether the inhibitory effects of the S100 proteins we observed were mediated by TLR4. Our results demonstrated that pretreating HUVECs with the TLR4 inhibitor before exposing them to recombinant human S100A8 and S100A9 in concentrations present in NSTEMI CD34-/CD31+ CM not only eliminated the attenuation in tube formation observed when HUVECs were exposed to S100A8 and

S100A9, but significantly improved tube formation compared to the positive control and the athlete condition. Interestingly, we also found that TAK-242 treatments alone significantly improved tube formation. TAK-242 acts by blocking TLR4 signaling of the intracellular domain (55). Some studies have found that this compound potentially suppresses ligand-independent signaling in addition to ligand-dependent signaling (73). TLR4-deficient mice have been found to exhibit impaired expression of pro-inflammatory cytokines, which can promote angiogenesis (114). This potentially explains our observed improvements in tube formation in the TAK-242 control condition and suggests that TLR4 inhibition in itself is a possible method for improving angiogenesis. Follow-up studies investigating the roll of TLR4 and the use of the drug TAK-242 to inhibit TLR4 signaling are necessary. As a whole, our results indicate that S100A8 and S100A9 secreted by CD34-/CD31+ CACs from NSTEMI patients impair HUVEC tube formation and that these actions appear to be mediated by TLR4.

In the current study we employed a gain of function experiment using recombinant S100A8 and S100A9 to mechanistically determine whether these proteins mediate the effects of the CM on tube formation. Future studies using immunoprecipitation, or other methods to block the function of S100A8 and S100A9 from the CM, would not only further confirm our findings, but would also help to identify the extent to which the improvements in tube formation are mediated by S100A8 and S100A9 ablation as opposed to suppression of TLR4 signaling. Similarly, small molecule inhibitors of TLR4 or other antibodies that exclusively block ligand-dependent signaling of TLR4 could be used to better understand the role of this receptor in angiogenesis and the extent to which S100A8 and S100A9 are contributing to the reduced tube formation observed as a result of CD34-/CD31+ CM from NSTEMIs.

Although S100A8, S100A9 and the S100A8/A9 heterodimer complex can each bind to TLR4, Wang et al. (2014) found that S100A8 preferentially binds to this receptor, with S100A9 having a preference for RAGE (162). Interestingly, when the concentrations of each of these proteins were estimated in the CM, there was proportionally more S100A8 present, suggesting the possibility that S100A8 is the major factor contributing to the observed negative effects on tube formation (118). S100A8 and S100A9 can function as monomers, as a heterodimer complex and as a heterotetramers with differences in their structure lending to functional diversity (153). Croce et al. (2009) demonstrated that the stability of these proteins is enhanced when functioning as a heterodimer complex (18), whereas several studies have experimentally tested the effects of each isolated protein (118, 162). Whereas methods to determine the physical structure of these proteins and their subsequent functions when secreted by CD34-/CD31+ CACs do not yet exist, future studies to test individual effects of S100A8 or S100A9 in the concentrations estimated to be present in the NSTEMI patients' CD34-/CD31+ CM in cell culture experiments would provide more information as to whether one is playing a more predominant role in the negative regulation of angiogenesis.

Upon binding to receptors present on endothelial cells, S100A8 and S100A9 are believed to modulate calcium signaling and cytoskeletal reorganization (18, 48, 153) through p38 and ERK1/2 signal pathways (162). One investigation into these signaling pathways in HUVEC cells found that S100A8/A9 caused endothelial barrier dysfunction increasing HUVEC monolayer permeability through RAGE and TLR4/p38/ERK1/2 signaling (162). As S100A8 and S100A9 are thought to be the predominant factors present in the CD34-/CD31+ CM that are affecting HUVEC tube formation, we sought to determine whether exposure to CD34-/CD31+ CM would activate similar signaling pathways in HUVECs and whether there would be differential response in NSTEMIs compared to athletes. We found that exposure to CD34-/CD31+ CM did not significantly alter p-ERK1/2, p-p38, p-NFκB p65 or IκBα in either group compared to the basal control. Additionally, we did not find any differences between groups with exposure to CD34-/CD31+ CM for any target protein. These results suggest that under our experimental conditions, factors within the CM for either group were not sufficient to activate these classically inflammatory signaling pathways.

We did observe a differential response to the drug treatments between groups for several targets. However, our results collectively do not support our other findings. TAK-242 treatments in the athlete group resulted in greater p-ERK1/2, p-p38 and p-NFκB p65 compared the NSTEMI group. Additionally, in the presence of the athletes' CD34-/CD31+ CM, TAK-242 treatments resulted in greater p-ERK1/2, and p-NFκB p65 compared to basal and CM only conditions. However, the same treatments used in the angiogenesis assay did not alter tube formation. Conversely, for the NSTEMI group, combined TAK-242 and FPS-ZM1 treatments resulted in greater p-ERK1/2 but reduced p-NFκB p65 compared to CM only conditions.

As the signaling experiments included in this study are part of an exploratory aim, it is likely that several components remain to be worked out before these data can be used in conjunction with our other findings. For instance, the 1-hour exposure time to the CD34-/CD31+ CM was selected based on prior work demonstrating that these signaling pathways reached peak activation after exposure to S100A8/A9 (162) for 1 hour, assuming that these proteins, if present in the CD34-/CD31+ CM, will result in peak signaling around the same timeframe. However, differences in the content of complete CD34-/CD31+ CM and isolated proteins may require a different timeframe for peak signaling in order to detect potential differences. Additionally, we were limited by the volume of CM we had to perform these experiments and as such, experiments were performed on a calculated number of harvested HUVECs, whereas other studies used cells in culture environments (162). Thus, differences in signaling and how drug treatments affect the HUVECs may exist in adherent vs. non-adherent cells. Cell signaling involves a series of complex actions. Consequently, in order to draw any concrete conclusions linking these signaling pathways to our observed results in tube formation, further work related to this aim is necessary.

Several studies have associated plasma S100A8/A9 with enhanced CV risk (5, 17). Surprisingly, we did not find any significant differences in plasma S100A8/A9 heterodimer complex between our endurance-trained subjects and NSTEMI patients. Most studies have examined plasma or serum S100A8/A9 concentrations as a predictor for CVD with few studies investigating systemic concentrations after an acute cardiac event (17, 43). Katashima et al. (2015) found that serum levels of S100A8/A9 peaked 3-5 days after symptom onset of acute myocardial infarction in STEMI patients (71). Thus, it appears that the timing of blood sampling after an acute event is important for detecting alterations in systemic concentrations of the S100A8/A9 heterodimer complex. In our current study, samples were taken within 24-72 hrs days of symptom onset, potentially leading to variable results.

A secondary aim of our study was to begin to characterize phenotypical traits of CD34-/CD31+ CACs from NSTEMI patients and endurance-trained group that may explain some of the observed differences in paracrine actions. Using a small subset of individuals from our study (n=3/group), we performed flow cytometry analyses and found that CD34-/CD31+ CACs from NSTEMI patients exhibited a lower percentage of markers for angiogenic T-cells (CD3) as well as monocytes (CD14) compared to the healthy endurance-trained athletes' CD34-/CD31+ CACs. Kim et al. (2014) also found lower levels of CD3 present in CAD patients' CD31+ CACs compared to healthy controls (76). However, contrary to our findings, they found higher levels of monocyte and macrophage markers in the CAD patients' cells. They suggest that the higher presence of these markers may indicate that the CACs from CAD patients are involved in more of an inflammatory rather than angiogenic response. However, several studies have found that circulating cells positive for both monocyte and endothelial markers exhibit angiogenic behaviors (32, 132) and are recruited to sites of vascular injury and contribute to angiogenesis (77). In the present study, we did not note any differences between groups in the expression of two angiogenic genes of interest. However the CD34-/CD31+ CACs from athletes expressed higher IL-1α mRNA levels. IL-1α is typically known for its inflammatory role (76, 93), however there is also evidence for IL-1α promoting angiogenesis by stimulating secretion of vascular endothelial growth factor (VEGF) from circulating cells (127, 157). Thus, these findings, coupled with our other results suggest a more angiogenic phenotype in the CD34-/CD31+ CACs of athletes compared to NSTEMI patients.

The primary goal of this study was to determine if functional and mechanistic differences exist between two extreme populations – the young endurance-trained athletes and the older CVD patients; therefore, we did not include an older healthy control group in the study. We know from our previous work, that differences in CD34-/CD31+ CAC secretion of S100A8 and S100A9 exist as a function of habitual physical activity in younger healthy adults. However, the inclusion of a third group of healthy adults without CVD that were age-matched to the NSTEMIs would be a valuable addition in order to further understand how differences in these paracrine actions occur throughout the lifespan.

Another limitation of this study is that the NSTEMI patients were currently taking a number of medications. Three athletes reported taking allergy or anxiety medications as needed and these medications were not taken for ≥ 12 hours prior to their tests and so likely did not affect any results. There are several medications that have been found to affect CAC number and function (28) and it is possible that some of the medications being taken by the NSTEMI patients affected our outcomes. We did not control for medications in the NSTEMI group because of the necessity of their habitual use in this population. Additionally, as our goal is to understand the function of CACs from patient populations that might benefit from autologous cell therapies, it is more physiologically relevant to study CACs from this group in the conditions at which they would be used if implementing these treatments.

Conclusions

In summary, we demonstrate, for the first time, that CD34-/CD31+ CM from NSTEMI patients impairs HUVEC tube formation and that these effects appear to be mediated by TLR4. We found that pro-inflammatory S100A8 and S100A9 content were substantially higher in the CM from NSTEMI patients compared to athletes. Experiments using recombinant S100A8 and S100A9 in the concentrations estimated to be present in the NSTEMI's CD34-CD31+ CM, but not the athlete's CM, further confirmed that these proteins are major contributors to the impaired tube formation and that the observed effects appear to be mediated by TLR4. Methods designed to alter the production and secretion of S100A8 and S100A9 from CVD patients' CD34-/CD31+ CACs to levels more similar to those of endurance-trained athlete's CACs may improve the efficacy of treatments employing these cells for use in clinical populations.

COMPETING INTERESTS

No competing interests are reported.

FUNDING

RQL was supported by NIH Predoctoral Institutional Training Grant T32AG000268 (to JMH) and by the University of Maryland Summer Research Fellowship (to RQL). This study was supported by NIH R21-HL98810 (to JMH), the University of Maryland Department of Kinesiology Graduate Research Initiative Fund (to RQL) and Baltimore VA Geriatric Research, Education, and Clinical Center (SJP).

ACKNOWLEDGEMENTS

We thank the study volunteers for their time and commitment to this study. We especially thank our Veteran patients and their families.

Table 4.1. List of Medications

Medication, *n* (%)	**NSTEMI**	**Athlete**
Statin	7 (54)	0
Blood Thinner	2 (15)	0
ACE Inhibitor	4 (31)	0
Beta Blocker	6 (46)	0
Anti-diabetic	7 (54)	0
Heartburn (GERD)	4 (31)	0
NSAID	11 (85)	0
Brochodilator	2 (15)	1 (8)
Nitrate	2 (15)	0
Prostaglandin E1 Agonist	1 (8)	0
SSNRI	1 (8)	0
Nonergot dopamine Agonist	1 (8)	0
PDE5 Inhibitor	2 (15)	0
Hypothyroidism	1 (8)	0
Calcium channel blocker	3 (23)	0
Diuretic	3 (23)	0
Carbonic anhydrase inhibitor	1 (8)	0
Ferrous Sulfate	2 (15)	0
Other blood pressure medication	2 (15)	0
Phosphate lowering medication	1 (8)	0
Angiotensin 2 Receptor Blocker	2 (15)	0
Antidepressant	2 (15)	2 (15)
Anti-epileptic	2 (15)	0
Alpha Blocker	1 (8)	0

Table 4.2. RT-PCR genes and primer sequences

Symbol	Gene name	Primer Sequence
S100A8	S100 calcium binding protein A8	F: CCCATCTTTATCACCAGAATGAG R: CCGAGTGTCCTCAGTATATCAG
S100A9	S100 calcium binding protein A9	F: CCTCCATGATGTGTTCTATGACC R: CAACACCTTCCACCAATACTCT
ANGPT1	angiopoietin 1	F: ACTTCCATCTTCACGATGTTGT R: GACTGTGCAGATGTATATCAAGC
FGF-2	fibroblast growth factor-2	F: CTTCATAGCCAGGTAACGGTT R: AGAAGAGCGACCCTCACA
IL1A	interleukin 1, alpha	F: TCTTCATCTTGGGCAGTCAC R: GCTGCTGCATTACATAATCTGG
TNF	tumor necrosis factor, alpha	F: TCAGCTTGAGGGTTTGCTAC R: TGCACTTTGGAGTGATCGG
GAPDH		F: TGTAGTTGAGGTCAATGAAGGG R: ACATCGCTCAGACACCATG

Table 4.3. Subject Characteristics

	Endurance Trained Athlete (n=13)	NSTEMI (n=13)
Age (yr)	28±2	71±3*
BMI (kg/m^2)	22±0.6	26±1*
Body Fat (%)	9.7±0.8	-
Absolute VO$_2$max (L/min)	4.5±0.1	-
Relative VO$_2$max (ml/kg/min)	63.2±1.7	-
SBP (mm Hg)	125±2.2	131±5
DBP (mm Hg)	69±2.3	71±3
MAP (mm Hg)	88±1.9	91±3
Glucose (mg/dl)	79±2	113±2*
Cholesterol (mg/dl)	172±9	169±18
HDL-C (mg/dl)	69±5	44±3*
LDL-C (mg/dl)	88±5	101±15
VLDL-C (mg/dl)	15±4	-
TC/HDL	2.5±0.2	3.8±0.4*
LDL/HDL	1.3±0.2	2.2±0.3*
Triglycerides (mg/dl)	55±6	118±24*

*Significantly different from athlete

Figure 4.1

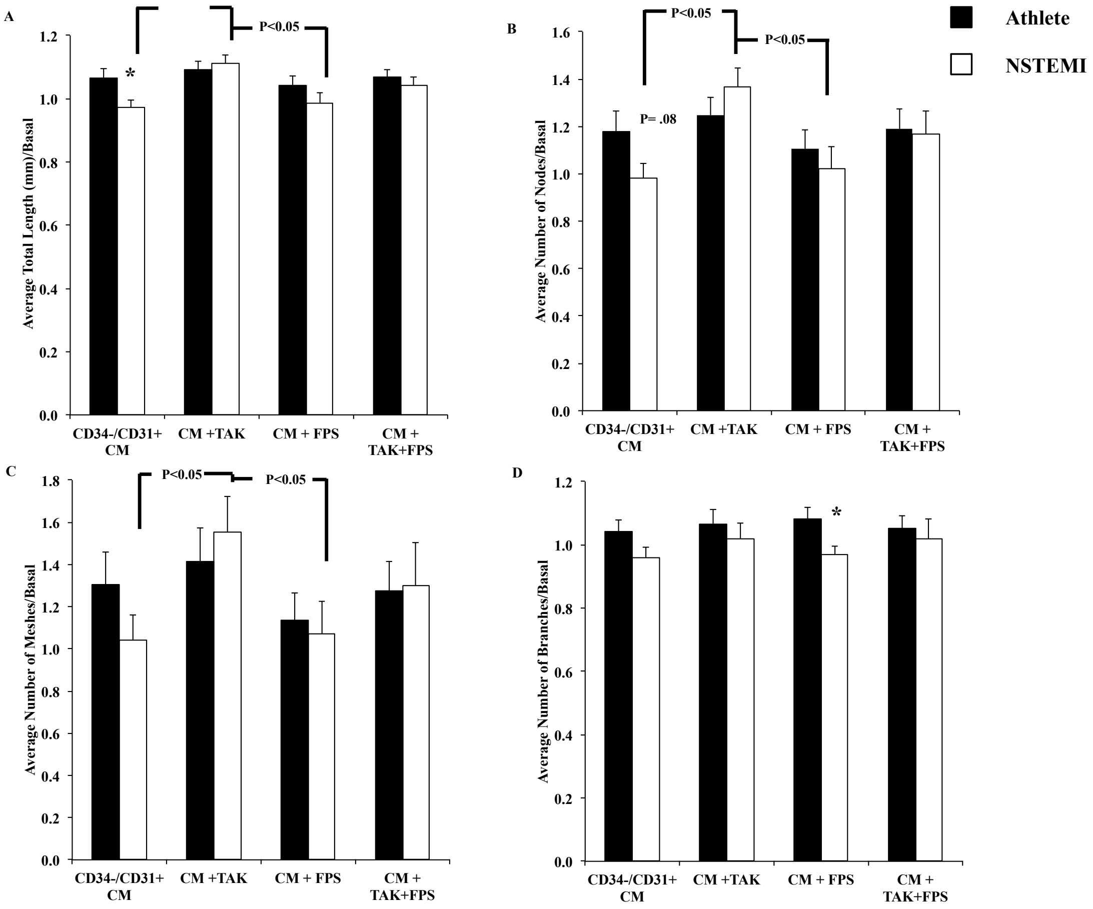


Figure 4.1. Quantification of HUVEC tube formation after culture with CM from CD34-/CD31+ CACs of endurance-trained athletes and NSTEMI patients with or without pretreatment of HUVECs with TAK242 (TLR4 signaling inhibitor) or FPS-ZM1 (RAGE antagonist). CACs were cultured in un-supplemented endothelial growth medium free of growth factors or serum, each at a density of 300,000 cells per well of a fibronectin-coated plate. Media was collected after 48hrs of culture from each cell type. HUVEC tube formation was assessed using Image J angiogenesis analyzer to quantify total length in millimeters (mm) (A) number of nodes (B), number of meshes (C) and number of branches (D). Results are presented as each condition normalized to the basal (EBM-2) condition to control for daily variability in HUVEC growth and passage number with each assay. Images were taken under 5x magnification. * Indicates statistically significant difference from endurance-trained athletes. Significance accepted at $P \leq 0.05$.

Figure 4.2

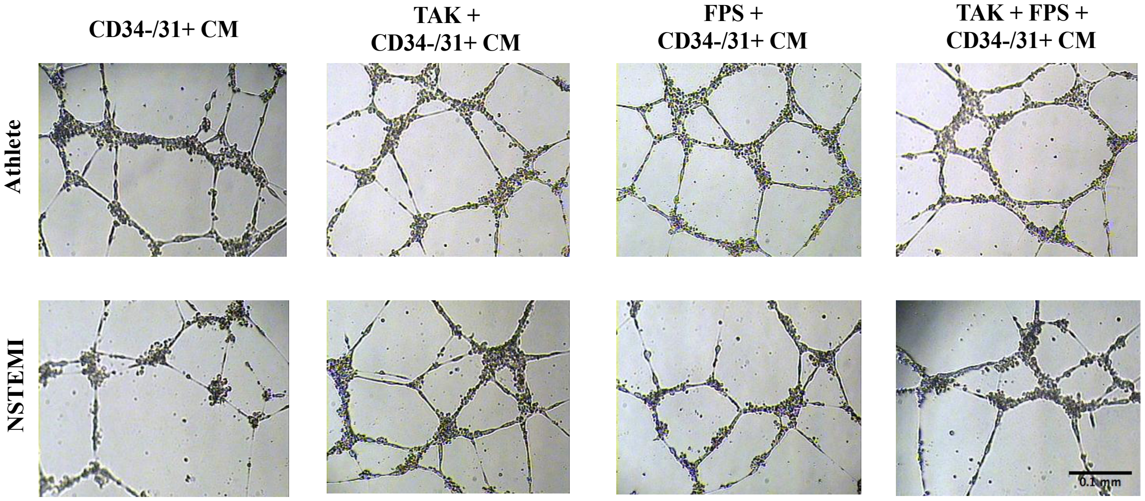

CD34-/31+ CM
TAK +
CD34-/31+ CM
FPS +
CD34-/31+ CM
TAK + FPS +
CD34-/31+ CM
Athlete
NSTEMI
0.1 mm

Figure 4.2. Representative images of HUVEC tube formation using CM from CD34-/CD31+ CACs of endurance trained athletes and NSTEMI patients with and without HUVEC pretreatment with TAK242 (TLR4 signaling inhibitor) and/or FPS-ZM1 (RAGE antagonist).

Figure 4.3

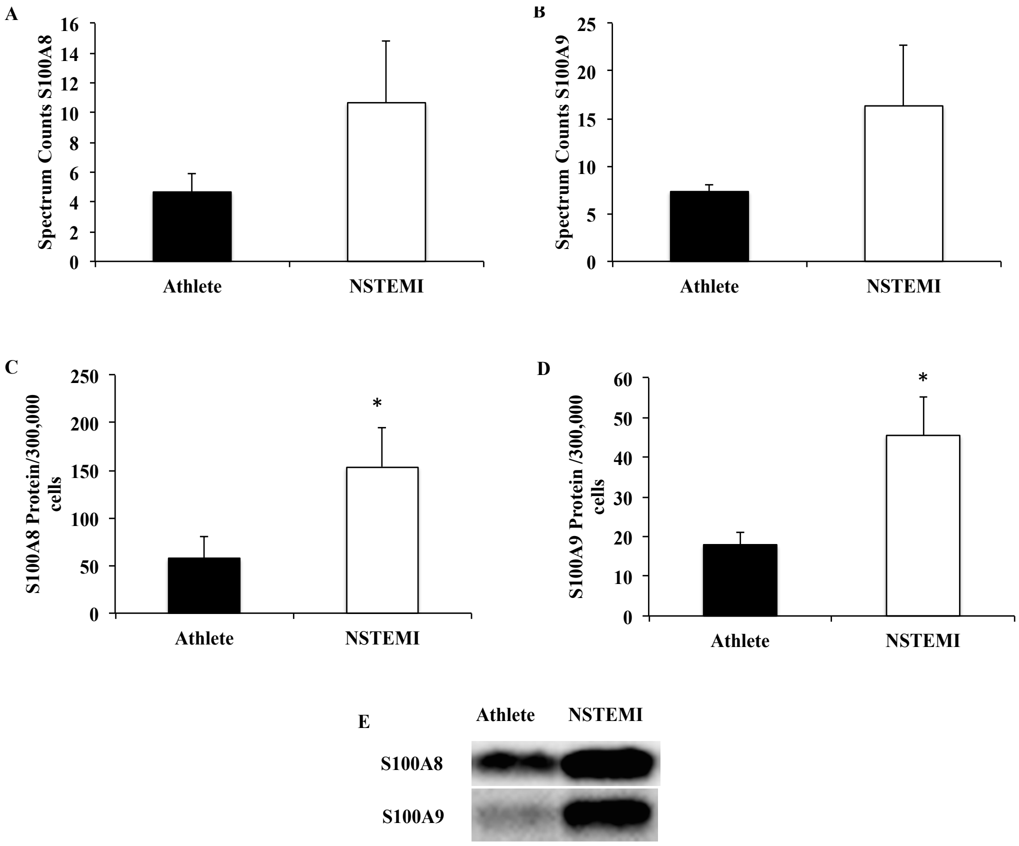


Figure 4.3. Semi-quantitative analysis of S100A8 (A) and S100A9 (B) in CM of cultured CD34-/CD31+ cells using mass spectrometry spectrum counts (n=3/group) to identify proteins present in conditioned medium. Quantitative analyses using immunoblotting for secreted S100A8 (C) and S100A9 (D) protein content in cultured CD34-/CD31+ cell CM from endurance-trained athletes and NSTEMI patients (n=10 per group). Values were normalized to cell number used to generate the CM (300,000 cells/well) as equal volumes of CM were loaded for western blot analysis. Representative blots for CD34-/CD31+ CM (E). * Indicates statistically different from endurance-trained athletes. Significance accepted at $P \leq 0.05$.

Figure 4.4

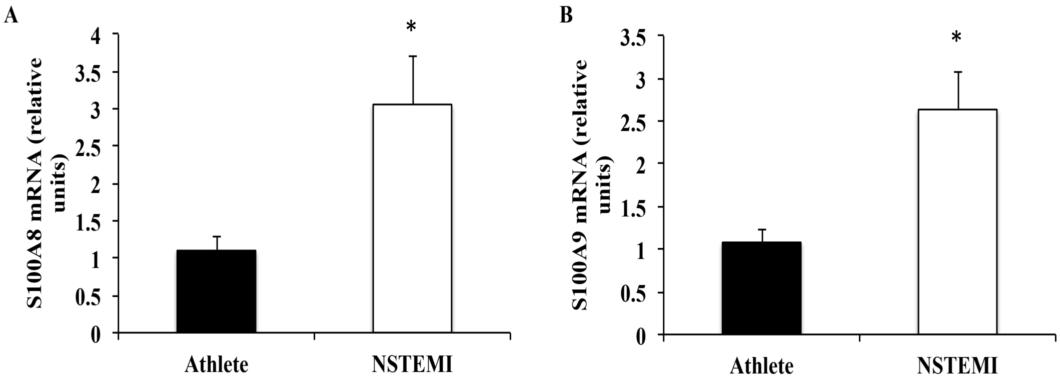


Figure 4.4. (A) S100A8 and (B) S100A9 real-time mRNA expression for freshly isolated CD34-/CD31+ circulating angiogenic cells (n=10). GAPDH was used to normalize all data. * Indicates statistically significant at $P<0.05$.

Figure 4.5

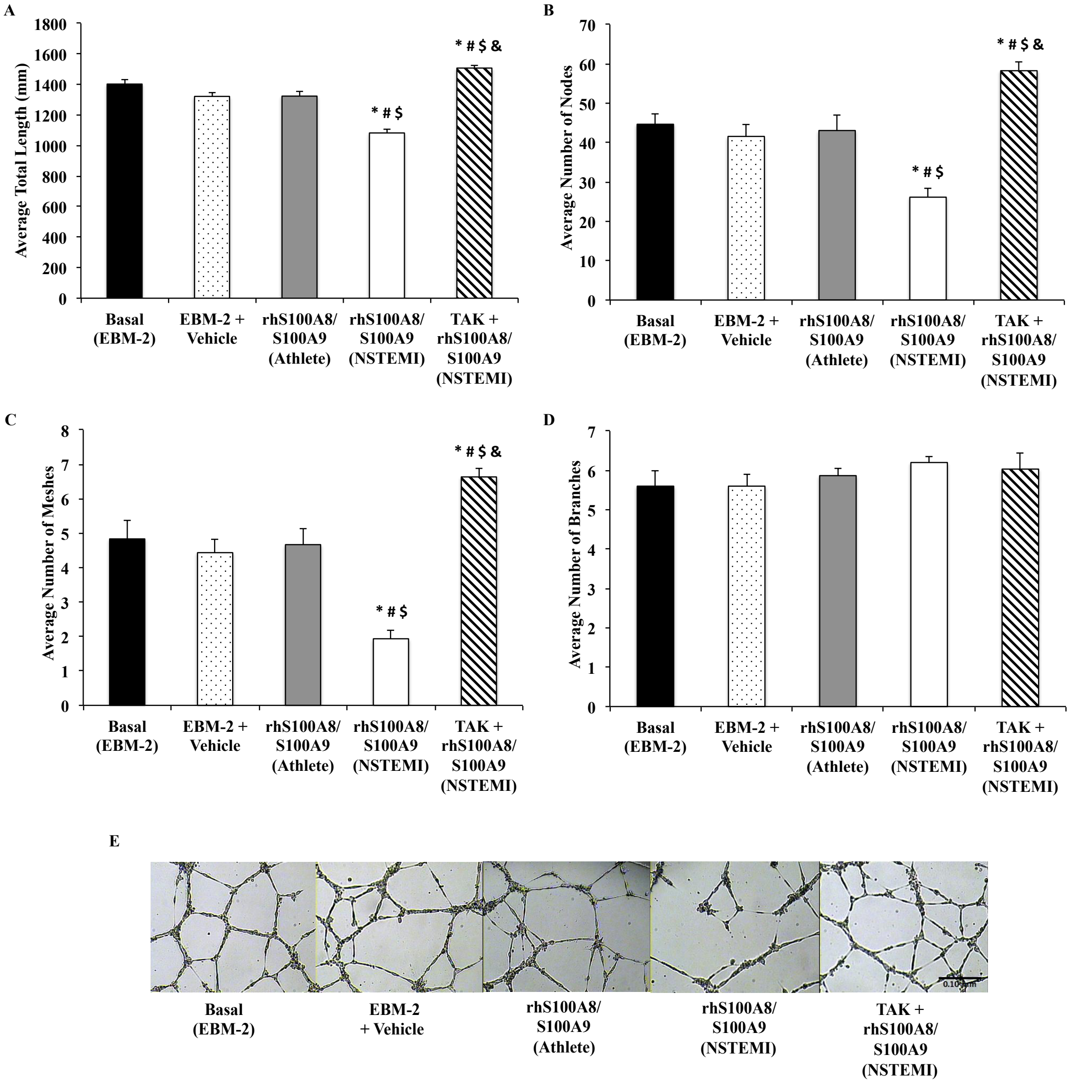


Figure 4.5. Effects of recombinant human S100A8 and S100A9 in concentrations estimated to be present in the CM of CD34-/CD31+ CAC from athletes (2.9 µg/mL and 0.8 µg/mL, respectively), NSTEMI patients (6.7 µg/mL and 1.4 µg/mL, respectively) and NSTEMI patients after pretreatment of HUVECs with TAK-242 on tube formation compared to basal media and vehicle conditions. HUVEC tube formation was assessed using Image J angiogenesis analyzer to quantify total length in millimeters (mm) (A) number of nodes (B), number of meshes (C) and number of branches (D) and representative images from each condition (E). In these experiments, each condition was assessed in samples collected from six independent cell culture wells from multiple culture plates collected on different days. All experiments were conducted on cells from the same passage number (P4). * Indicates statistically different basal condition. # Indicates statistically different than rhS100 athlete condition. $ Indicates statistically different than vehicle condition. & Indicates statistically different than rhS10 NSTEMI condition. Significance accepted at $P \leq 0.05$.

Figure 4.6

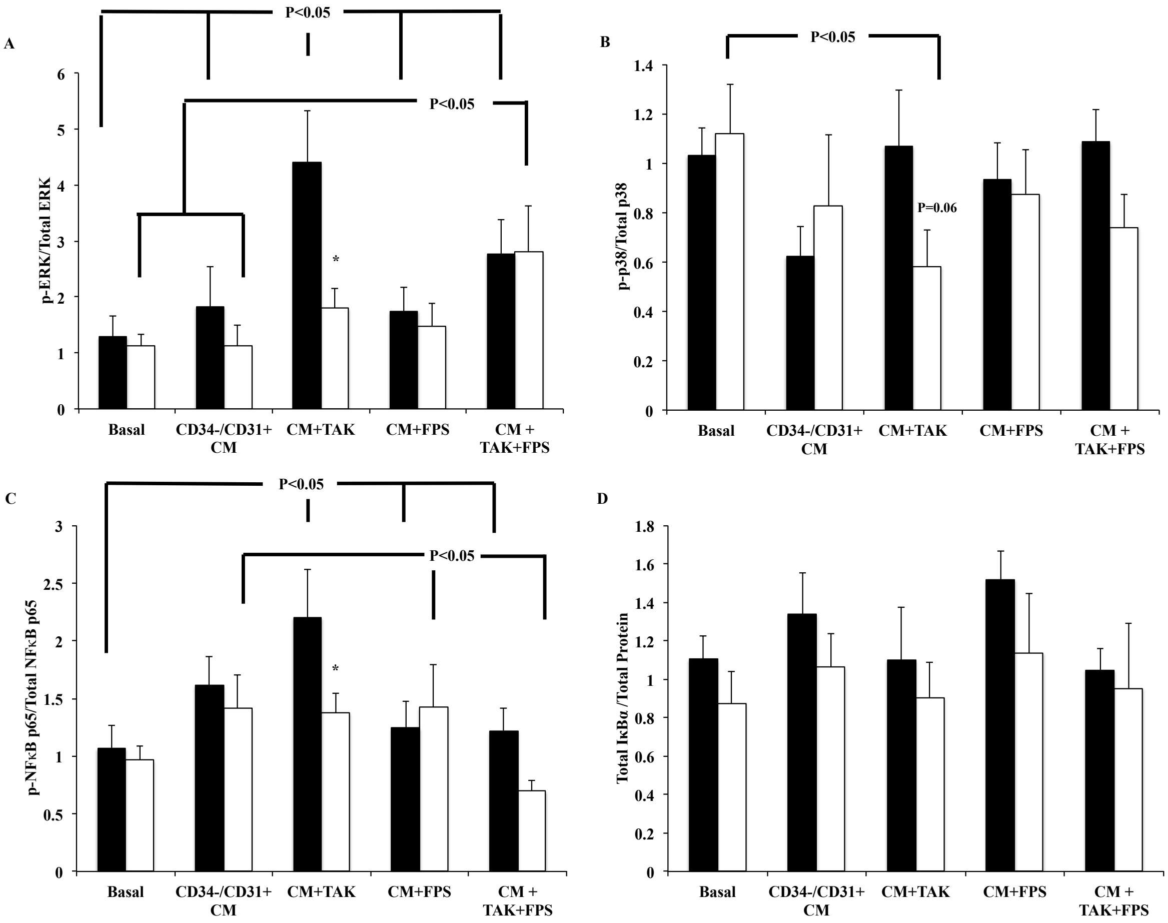


Figure 4.6. Immunoblot analyses of HUVEC after 1 hr exposure to CM from athletes or NSTEMI CD34-/CD31+ CACs with or without pretreatment with TAK-242 or FPS-ZM1 (n=6/group). HUVECs were assessed for phosphorylation of (A) ERK1/2, (B) p38, and (C) NFκB p65 subunit or degradation of IκBα (D). The average basal condition from each membrane served as a blot control and values are presented as phospho/total target protein after normalizing to total protein, or normalized to total protein when no phosphorylation event was assessed. * Indicates statistically different from endurance-trained athletes. Significance accepted at $P \leq 0.05$.

Figure 4.7

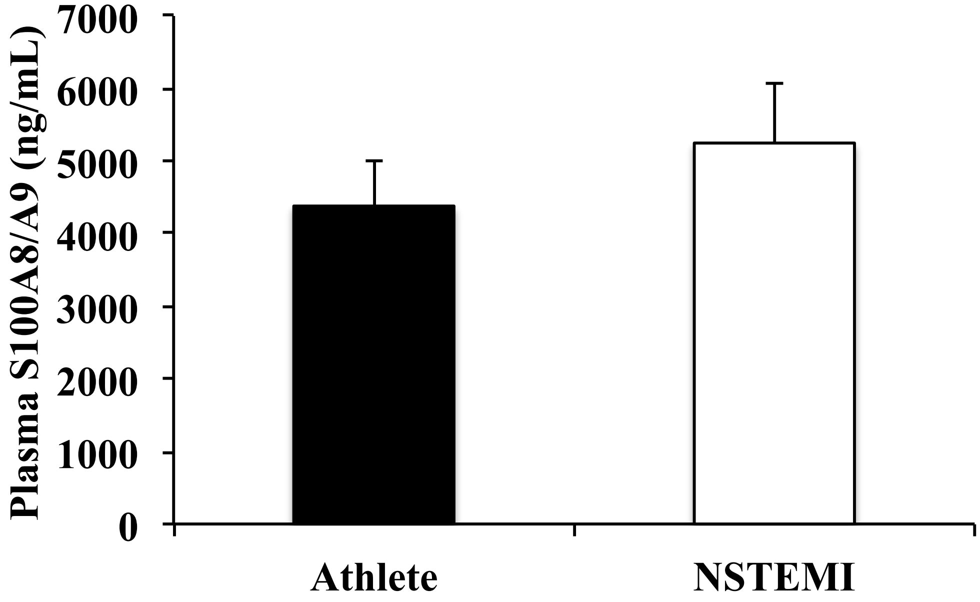


Figure 4.7. Plasma S100A8/A9 heterodimer complex levels from fasting venous blood samples of endurance-trained athletes and NSTEMI patients (n=13/group).

Figure 4.8

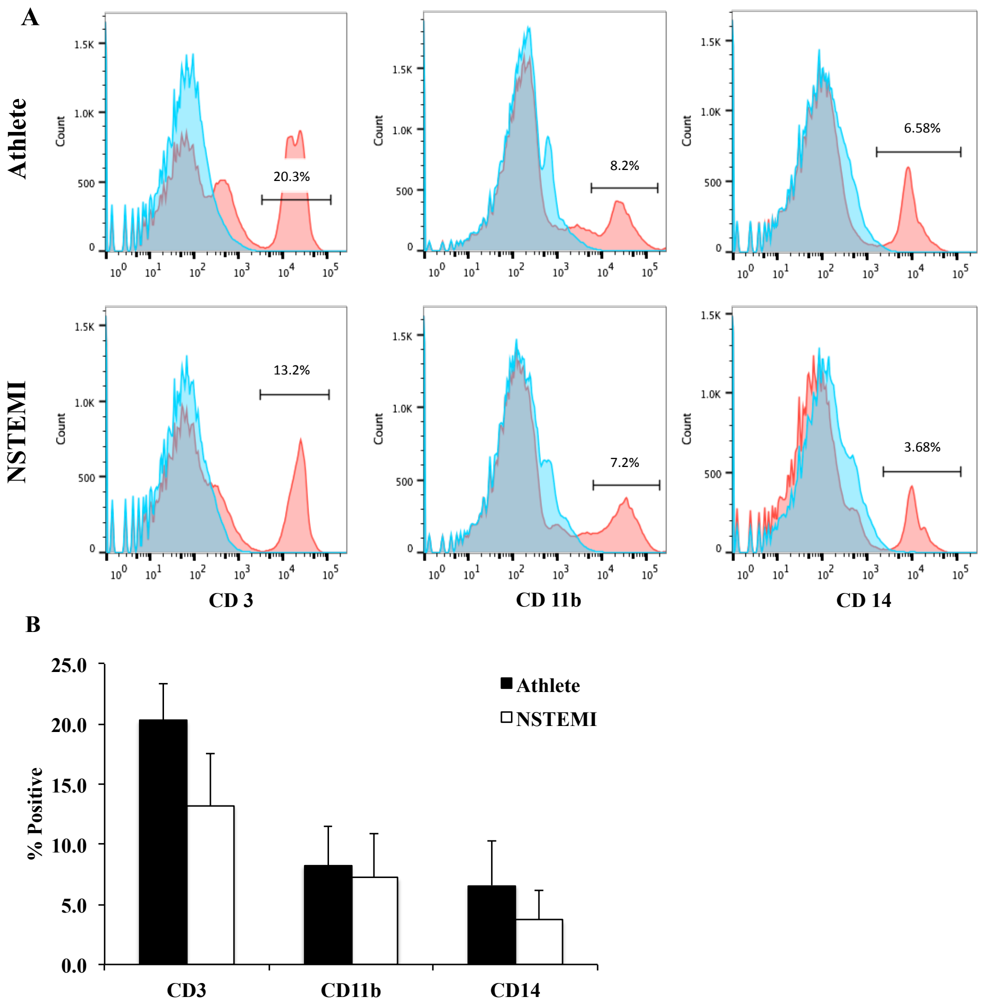


Figure 4.8. Characteristics of immunomagentically sorted CD34-/31+ CACs. A) Representative plots of athlete or NSTEMI CD34-/31+ CACs analyzed by flow cytometry for presence of CD3, CD11b and CD14. Blue area indicate isotype control and red areas indicate specific antibodies B) Mean percent positive CD3, CD11b and CD14 in athlete vs. NSTEMI selected CD34-/31+ CACs (n=3/group).

Figure 4.9

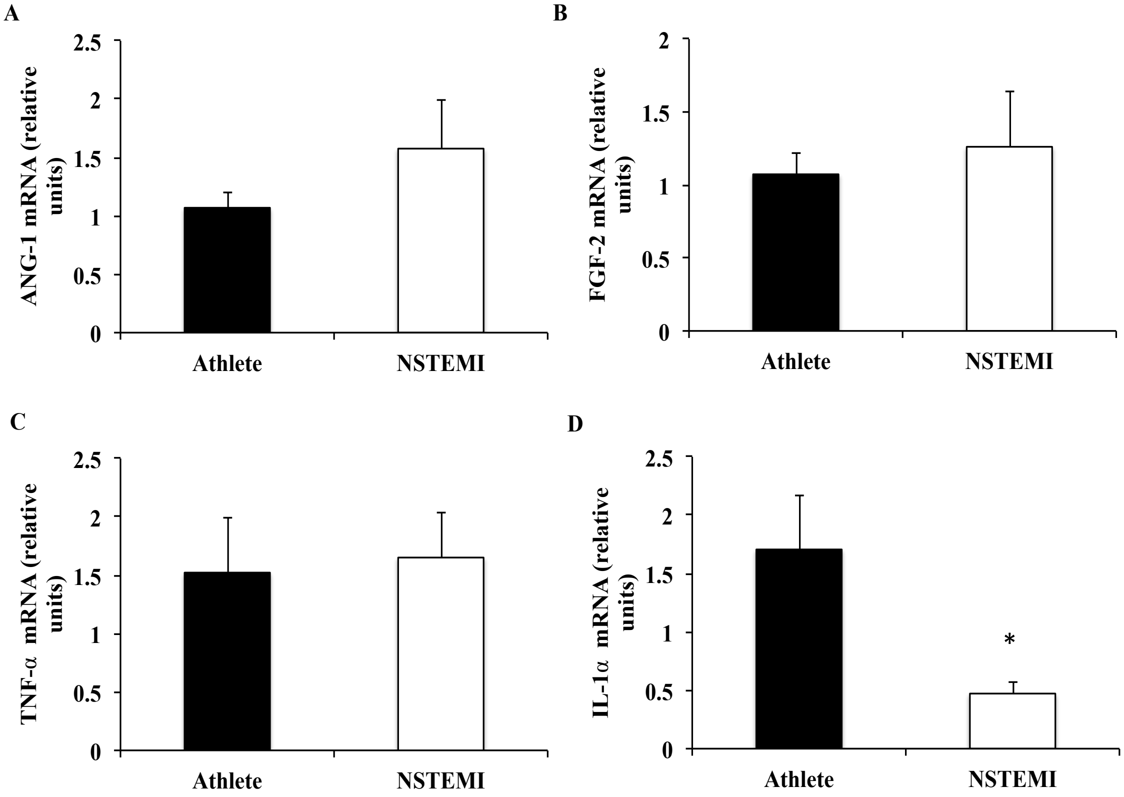


Figure 4.9. (A) angiopoieten-1, (B) FGF-2, (C) TNF-α and (D) IL-1α real-time mRNA expression for freshly isolated CD34-/CD31+ circulating angiogenic cells (n=10). GAPDH was used to normalize all data. * Indicates statistically significant at P<0.05.

Supplemental Table I. Proteins identified in the secretome of CD34-/31+ CACs by shotgun proteomics

Identified Proteins	Entry Name	Accession Number	MW	Average Number Total Spectra (n=3/group)	
				Athlete CD34-/31+	NSTEMI CD34-/31+
Isoform 2 of Filamin-A	FLNA_HUMAN	P21333-2	280 kDa	27.3	43.3
Thrombospondin-1	TSP1_HUMAN	P07996	129 kDa	33.7	56.0
Isoform 3 of Fibronectin	FINC_HUMAN	P02751-3	259 kDa	27.3	36.0
Myosin-9	MYH9_HUMAN	P35579	227 kDa	23.7	27.7
Serum albumin	ALBU_HUMAN	P02768	69 kDa	31.7	37.0
Protein S100-A9	S10A9_HUMAN	P06702	14 kDa	7.3	16.3
Talin-1	TLN1_HUMAN	Q9Y490	270 kDa	19.3	25.0
Plastin-2	PLSL_HUMAN	P13796	70 kDa	14.0	14.3
Actin, cytoplasmic 2	ACTG_HUMAN	P63261	42 kDa	15.7	16.0
Alpha-actinin-1	ACTN1_HUMAN	P12814	103 kDa	11.7	21.3
Alpha-enolase	ENOA_HUMAN	P06733	47 kDa	6.0	9.0
Triosephosphate isomerase	TPIS_HUMAN	P60174	31 kDa	7.3	12.3
Transketolase	TKT_HUMAN	P29401	68 kDa	3.7	9.7
Protein S100-A8	S10A8_HUMAN	P05109	11 kDa	4.7	10.7
Lactotransferrin	E7ER44_HUMAN	E7ER44	78 kDa	11.0	14.7
Fibrinogen gamma chain	C9JC84_HU	C9JC84	52	11.3	15.7

	MAN		kDa		
Catalase	CATA_HUMAN	P04040	60 kDa	5.0	9.7
Moesin	MOES_HUMAN	P26038	68 kDa	5.7	6.7
Fibrinogen beta chain	FIBB_HUMAN	P02675	56 kDa	12.0	13.7
Isoform H7 of Myeloperoxidase	PERM_HUMAN	P05164-3	87 kDa	1.0	10.0
Vimentin	VIME_HUMAN	P08670	54 kDa	5.3	4.7
Fibrinogen alpha chain	FIBA_HUMAN	P02671	95 kDa	10.7	15.3
Isoform 1 of Vinculin	VINC_HUMAN	P18206-2	117 kDa	11.7	9.3
Pyruvate kinase PKM	KPYM_HUMAN	P14618	58 kDa	3.3	5.0
Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	P04406	36 kDa	2.7	5.7
14-3-3 protein zeta/delta	1433Z_HUMAN	P63104	28 kDa	7.3	7.3
Isoform 2 of Phosphoglycerate kinase 1	PGK1_HUMAN	P00558-2	41 kDa	4.7	5.7
Fructose-bisphosphate aldolase A	ALDOA_HUMAN	P04075	39 kDa	2.7	5.0
Isoform 5 of Tropomyosin alpha-3 chain	TPM3_HUMAN	P06753-5	29 kDa	7.3	6.0
Calmodulin (Fragment)	H0Y7A7_HUMAN	H0Y7A7	21 kDa	4.0	5.0
Profilin-1	PROF1_HUMAN	P07737	15 kDa	5.7	6.7
SH3 domain-binding glutamic acid-rich-like protein 3	A0A087WV23_HUMAN	A0A087WV23	24 kDa	5.7	5.0
Hemoglobin subunit beta	HBB_HUMAN	P68871	16 kDa	2.0	1.7
Cofilin-1	COF1_HUMAN	P23528	19 kDa	6.3	6.0
Latent-transforming growth factor beta-	LTBP1_HU	Q14766	187	6.7	7.0

binding protein 1	MAN		kDa		
Isoform 3 of Gelsolin	GELS_HUMAN	P06396-3	82 kDa	5.3	7.7
Superoxide dismutase [Cu-Zn]	SODC_HUMAN	P00441	16 kDa	4.3	5.3
Histone H4	H4_HUMAN	P62805	11 kDa	4.0	3.7
Heat shock 70 kDa protein 1A/1B	HSP71_HUMAN	P08107	70 kDa	1.7	3.3
Histone H2B type 1-L	H2B1L_HUMAN	Q99880	14 kDa	2.0	3.7
Heat shock cognate 71 kDa protein	HSP7C_HUMAN	P11142	71 kDa	3.3	4.0
Lymphocyte-specific protein 1	LSP1_HUMAN	P33241	37 kDa	3.0	6.3
Zyxin	ZYX_HUMAN	Q15942	61 kDa	4.0	7.0
Transaldolase	TALDO_HUMAN	P37837	38 kDa	1.7	3.7
Platelet basic protein 3	CXCL7_HUMAN	P02775	14 kDa	5.3	5.7
SH3 domain-binding glutamic acid-rich-like protein	SH3L1_HUMAN	O75368	13 kDa	3.7	3.0
Tropomyosin alpha-4 chain	SH3L1_HUMAN	P67936	29 kDa	9.0	8.3
Rab GDP dissociation inhibitor beta	GDIB_HUMAN	P50395	51 kDa	2.3	4.3
Isoform 2 of Transgelin-2	TAGL2_HUMAN	P37802	24 kDa	4.7	5.3
Bridging integrator 2	A0A087X188_HUMAN	A0A087X188	65 kDa	7.0	4.3
Multimerin-1	MMRN1_HUMAN	Q13201	138 kDa	4.7	5.3
Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	P62937	18 kDa	4.3	4.0
Peptidyl-prolyl cis-trans isomerase B	PPIB_HUMAN	P23284	24 kDa	4.7	6.3
Hemoglobin subunit alpha	HBA_HUMA	P69905	15	1.7	1.0

	N		kDa		
Adenylyl cyclase-associated protein	D3DPU2_HUMAN	D3DPU2	52 kDa	5.0	6.0
Glucose-6-phosphate isomerase	G6PI_HUMAN	P06744	63 kDa	1.0	4.7
Ig gamma-1 chain C region	A0A087X1C7_HUMAN	A0A087X1C7	50 kDa	4.7	4.7
Serotransferrin	TRFE_HUMAN	P02787	77 kDa	4.0	5.3
Histone H1.2	H12_HUMAN	P16403	21 kDa	3.0	4.3
Protein DJ-1	PARK7_HUMAN	Q99497	20 kDa	2.0	4.3
Leukocyte elastase inhibitor	ILEU_HUMAN	P30740	43 kDa	3.7	4.0
Isoform Sap-mu-6 of Prosaposin	SAP_HUMAN	P07602-2	58 kDa	3.7	4.0
Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_HUMAN	P22626	37 kDa	1.3	2.7
Neutrophil gelatinase-associated lipocalin	X6R8F3_HUMAN	X6R8F3	23 kDa	2.3	6.0
Alpha-fetoprotein	J3KMX3_HUMAN	J3KMX3	70 kDa	2.7	3.0
Platelet glycoprotein Ib alpha chain	GP1BA_HUMAN	P07359	72 kDa	3.0	5.7
Matrix metalloproteinase-9	MMP9_HUMAN	P14780	78 kDa	5.0	6.3
Carbonic anhydrase 2	CAH2_HUMAN	P00918	29 kDa	3.7	6.0
Brain acid soluble protein 1	BASP1_HUMAN	P80723	23 kDa	4.0	3.7
Ig kappa chain C region	A0A087X130_HUMAN	A0A087X130	25 kDa	3.3	4.0
Cathepsin S	CATS_HUMAN	P25774	37 kDa	3.0	3.7
HCG1780554	Q5T4B6_HUMAN	Q5T4B6	5 kDa	4.0	4.3
Alpha-actinin-4	ACTN4_HU	O43707	105	6.0	11.3

	MAN		kDa		
von Willebrand factor	VWF_HUMAN	P04275	309 kDa	2.0	4.7
Serum deprivation-response protein	SDPR_HUMAN	O95810	47 kDa	4.7	3.7
Isoform 3 of L-lactate dehydrogenase A chain	LDHA_HUMAN	P00338-3	40 kDa	1.3	2.3
Coronin-1A	COR1A_HUMAN	P31146	51 kDa	0.7	3.3
Annexin A1	ANXA1_HUMAN	P04083	39 kDa	1.3	3.0
Glutathione S-transferase P	GSTP1_HUMAN	P09211	23 kDa	2.7	2.3
Isoform Smooth muscle of Myosin light polypeptide 6	MYL6_HUMAN	P60660-2	17 kDa	3.3	3.0
Histone H2A type 3	H2A3_HUMAN	Q7L7L0	14 kDa	2.3	1.7
L-lactate dehydrogenase B chain	LDHB_HUMAN	P07195	37 kDa	3.3	2.7
Coagulation factor XIII A chain	F13A_HUMAN	P00488	83 kDa	2.7	5.7
Histone H1.5	H15_HUMAN	P16401	23 kDa	1.7	3.7
Cathepsin D	CATD_HUMAN	P07339	45 kDa	2.0	4.3
Tubulin alpha-1B chain	TBA1B_HUMAN	P68363	50 kDa	1.3	2.3
Cathepsin B	CATB_HUMAN	P07858	38 kDa	2.0	5.0
Granulins	GRN_HUMAN	P28799	64 kDa	0.7	4.0
Transitional endoplasmic reticulum ATPase	TERA_HUMAN	P55072	89 kDa	1.0	1.7
Platelet glycoprotein V	GPV_HUMAN	P40197	61 kDa	2.7	4.7
Caldesmon	CALD1_HUMAN	Q05682	93 kDa	3.7	3.3
Serine/threonine-protein phosphatase	CPPED_HU	Q9BRF8	36	0.7	3.7

CPPED1	MAN		kDa		
Adenylate kinase 2, mitochondrial	KAD2_HUMAN	P54819	26 kDa	2.0	2.3
Protein disulfide-isomerase A3	PDIA3_HUMAN	P30101	57 kDa	4.0	3.7
PDZ and LIM domain protein 1	PDLI1_HUMAN	O00151	36 kDa	2.0	3.0
Integrin alpha-IIb	ITA2B_HUMAN	P08514	113 kDa	2.0	4.3
Vitamin D-binding protein	D6RF35_HUMAN	D6RF35	53 kDa	2.3	2.3
Src substrate cortactin	SRC8_HUMAN	Q14247	62 kDa	3.3	3.3
6-phosphogluconate dehydrogenase, decarboxylating	6PGD_HUMAN	P52209	53 kDa	1.3	2.0
Heterogeneous nuclear ribonucleoprotein M	A0A087X0X3_HUMAN	A0A087X0X3	78 kDa	1.0	1.0
SPARC	D3DQH8_HUMAN	D3DQH8	36 kDa	4.0	5.0
Phosphatidylethanolamine-binding protein 1	PEBP1_HUMAN	P30086	21 kDa	1.3	2.3
Rho GDP-dissociation inhibitor 1 (Fragment)	J3KTF8_HUMAN	J3KTF8	22 kDa	1.0	2.7
Inter-alpha-trypsin inhibitor heavy chain H2	A0A087WTE1_HUMAN	A0A087WTE1	107 kDa	2.7	2.0
Isoform 10 of Calpastatin	ICAL_HUMAN	P20810-10	82 kDa	1.0	0.7
Rho GDP-dissociation inhibitor 2	GDIR2_HUMAN	P52566	23 kDa	2.0	1.0
Isoform 3 of Glutathione reductase, mitochondrial	GSHR_HUMAN	P00390-4	50 kDa	0.7	1.7
Annexin A2	ANXA2_HUMAN	P07355	39 kDa	1.3	0.3
Calreticulin	CALR_HUMAN	P27797	48 kDa	2.0	2.0
Chloride intracellular channel protein 1	CLIC1_HUMAN	O00299	27 kDa	0.0	1.0
Purine nucleoside phosphorylase	PNPH_HUM	P00491	32	0.7	3.3

	AN		kDa		
Coactosin-like protein	COTL1_HUMAN	Q14019	16 kDa	1.3	2.0
Splicing factor, proline- and glutamine-rich	SFPQ_HUMAN	P23246	76 kDa	0.3	1.0
Alpha-2-macroglobulin	A2MG_HUMAN	P01023	163 kDa	2.0	1.0
DNA-(apurinic or apyrimidinic site) lyase	APEX1_HUMAN	P27695	36 kDa	1.3	1.7
Beta-2-microglobulin	B2MG_HUMAN	P61769	14 kDa	1.3	2.0
14-3-3 protein epsilon	1433E_HUMAN	P62258	27 kDa	4.3	4.0
Histone H3.2	H32_HUMAN	Q71DI3	15 kDa	0.3	2.0
Leukotriene A-4 hydrolase	LKHA4_HUMAN	P09960	69 kDa	1.0	1.7
Phosphoglycerate mutase 1	PGAM1_HUMAN	P18669	29 kDa	1.0	1.3
Lamina-associated polypeptide 2, isoform alpha	LAP2A_HUMAN	P42166	75 kDa	0.7	0.7
Ubiquitin-40S ribosomal protein S27a	RS27A_HUMAN	P62979	18 kDa	2.0	2.3
Isoform Short of 14-3-3 protein beta/alpha	1433B_HUMAN	P31946	28 kDa	3.7	2.7
Proteasome subunit alpha type-7	PSA7_HUMAN	O14818	28 kDa	1.7	1.0
Isoform 2 of F-actin-capping protein subunit beta	CAPZB_HUMAN	P47756	31 kDa	0.0	1.3
Neutrophil elastase	ELNE_HUMAN	P08246	29 kDa	0.0	3.0
Complement C3	CO3_HUMAN	P01024	187 kDa	0.7	1.3
Putative elongation factor 1-alpha-like 3	EF1A3_HUMAN	Q5VTE0	50 kDa	1.0	1.0
Xaa-Pro dipeptidase	PEPD_HUMAN	P12955	55 kDa	0.7	0.7
Serglycin	SRGN_HUM	P10124	18	1.0	2.3

	AN		kDa		
Ras-related protein Rap-1b	RAP1B_HUMAN	P61224	21 kDa	0.7	2.3
Superoxide dismutase [Mn], mitochondrial	SODM_HUMAN	P04179	25 kDa	1.0	1.3
Myristoylated alanine-rich C-kinase substrate	A0A087WZH7_HUMAN	A0A087WZH7	32 kDa	2.3	1.7
Apoptosis-associated speck-like protein containing a CARD	ASC_HUMAN	Q9ULZ3	22 kDa	0.0	1.0
Isoform 3 of Drebrin-like protein	DBNL_HUMAN	Q9UJU6-3	49 kDa	1.0	1.3
Peroxiredoxin-6	PRDX6_HUMAN	P30041	25 kDa	2.3	2.3
Platelet factor 4	PLF4_HUMAN	P02776	11 kDa	1.3	2.7
P-selectin	Q5R341_HUMAN	Q5R341	84 kDa	1.3	4.0
Actin-related protein 3	ARP3_HUMAN	P61158	47 kDa	0.3	1.0
Cytochrome c (Fragment)	C9JFR7_HUMAN	C9JFR7	11 kDa	1.3	2.7
Ras suppressor protein 1	RSU1_HUMAN	Q15404	32 kDa	1.7	1.7
78 kDa glucose-regulated protein	GRP78_HUMAN	P11021	78 kDa	1.7	5.0
Ig mu chain C region	A0A087X2C0_HUMAN	A0A087X2C0	64 kDa	2.0	0.7
Ras-related protein Rab-11B	RB11B_HUMAN	Q15907	24 kDa	3.7	1.3
Actin, alpha cardiac muscle 1	ACTC_HUMAN	P68032	42 kDa	7.7	4.3
WD repeat-containing protein 1	WDR1_HUMAN	O75083	66 kDa	0.7	1.0
Adenosine deaminase CECR1	B4E3Q4_HUMAN	B4E3Q4	54 kDa	1.3	0.7
Protein S100-A12	S10AC_HUMAN	P80511	11 kDa	0.7	1.3
Ig heavy chain V-III region GAL	HV320_HU	P01781	13	0.0	0.7

	MAN		kDa		
Monocyte differentiation antigen CD14	CD14_HUMAN	P08571	40 kDa	0.7	0.7
Isoform 2 of Fermitin family homolog 3	URP2_HUMAN	Q86UX7-2	75 kDa	2.7	2.3
14-3-3 protein gamma	1433G_HUMAN	P61981	28 kDa	3.7	2.3
Twinfilin-2	TWF2_HUMAN	Q6IBS0	40 kDa	0.7	1.3
Immunoglobulin lambda-like polypeptide 5	A0A087WU42_HUMAN	A0A087WU42	25 kDa	1.0	1.3
Far upstream element-binding protein 2	FUBP2_HUMAN	Q92945	73 kDa	0.3	0.7
Nidogen-1	NID1_HUMAN	P14543	136 kDa	0.7	1.3
Nexilin	NEXN_HUMAN	Q0ZGT2	81 kDa	0.3	2.7
Antithrombin-III	ANT3_HUMAN	P01008	53 kDa	1.0	1.7
Elongation factor 1-gamma	EF1G_HUMAN	P26641	50 kDa	1.3	1.0
Pleckstrin	PLEK_HUMAN	P08567	40 kDa	2.0	2.3
Proteasome subunit alpha type-2	PSA2_HUMAN	P25787	26 kDa	0.3	1.0
Metalloproteinase inhibitor 1	Q5H9A7_HUMAN	Q5H9A7	16 kDa	0.3	0.7
Legumain	LGMN_HUMAN	Q99538	49 kDa	0.7	2.3
LIM and SH3 domain protein 1	LASP1_HUMAN	Q14847	30 kDa	1.7	1.0
Isoform 2 of L-selectin	LYAM1_HUMAN	P14151	44 kDa	1.0	0.0
Growth factor receptor-bound protein 2	GRB2_HUMAN	P62993	25 kDa	0.3	0.7
Isoform 2 of Transmembrane protein 40	TMM40_HUMAN	Q8WWA1-2	22 kDa	1.3	2.7
Macrophage-capping protein	CAPG_HUM	P40121	38	1.3	0.3

	AN		kDa		
Isoform 2 of WD repeat-containing protein 44	WDR44_HUMAN	Q5JSH3-2	100 kDa	1.0	1.0
Ras-related protein Rab-27B	RB27B_HUMAN	O00194	25 kDa	1.7	2.3
Ras-related protein Rab-7a	RAB7A_HUMAN	P51149	23 kDa	1.0	0.7
Malate dehydrogenase, mitochondrial	MDHM_HUMAN	P40926	36 kDa	0.3	0.3
Proteasome subunit beta type-3	PSB3_HUMAN	P49720	23 kDa	0.3	0.7
Fructose-1,6-bisphosphatase 1	F16P1_HUMAN	P09467	37 kDa	0.3	0.3
Coagulation factor V	FA5_HUMAN	P12259	252 kDa	0.7	1.0
Integrin beta-3	ITB3_HUMAN	P05106	87 kDa	1.0	1.7
Serine/threonine-protein kinase 10	STK10_HUMAN	O94804	112 kDa	0.0	1.3
Protein CDV3 homolog	CDV3_HUMAN	Q9UKY7	27 kDa	0.3	1.0
HLA class I histocompatibility antigen, B-73 alpha chain	1B73_HUMAN	Q31612	40 kDa	0.7	1.0
Tissue alpha-L-fucosidase	FUCO_HUMAN	P04066	54 kDa	1.0	0.7
Pigment epithelium-derived factor	PEDF_HUMAN	P36955	46 kDa	1.3	1.0
Serine/threonine-protein phosphatase 6 regulatory subunit 1	PP6R1_HUMAN	Q9UPN7	97 kDa	1.3	0.7
Myeloblastin	PRTN3_HUMAN	P24158	28 kDa	0.0	1.3
Isoform 3 of CD44 antigen	CD44_HUMAN	P16070-3	78 kDa	1.0	0.7
Fructose-bisphosphate aldolase C	ALDOC_HUMAN	P09972	39 kDa	0.7	1.0
Nuclease-sensitive element-binding protein 1	A0A087X1S2_HUMAN	A0A087X1S2	34 kDa	1.0	1.0
Actin-related protein 2/3 complex	ARPC2_HU	O15144	34	0.3	1.0

subunit 2	MAN		kDa		
Stromal interaction molecule 1	STIM1_HUMAN	Q13586	77 kDa	2.3	2.3
High mobility group protein B1	HMGB1_HUMAN	P09429	25 kDa	0.0	1.3
Isoform 3 of Malate dehydrogenase, cytoplasmic	MDHC_HUMAN	P40925-3	39 kDa	0.3	1.0
Ig alpha-2 chain C region	IGHA2_HUMAN	P01877	37 kDa	2.0	0.7
Alpha-1-acid glycoprotein 1	A1AG1_HUMAN	P02763	24 kDa	0.3	1.3
Calponin-2	CNN2_HUMAN	Q99439	34 kDa	1.0	1.3
Microtubule-associated protein RP/EB family member 1	MARE1_HUMAN	Q15691	30 kDa	0.3	1.3
Rubber elongation factor protein	HEVBR_HUMAN	P15252	15 kDa	0.7	0.7
26S proteasome non-ATPase regulatory subunit 2	PSMD2_HUMAN	Q13200	100 kDa	0.3	0.7
Isoform 4 of Tyrosine-protein phosphatase non-receptor type 6	PTN6_HUMAN	P29350-4	70 kDa	0.0	0.7
Protein S100-A11	S10AB_HUMAN	P31949	12 kDa	0.3	0.7
Trem-like transcript 1 protein	TRML1_HUMAN	Q86YW5	33 kDa	0.7	1.0
Non-secretory ribonuclease	RNAS2_HUMAN	P10153	18 kDa	0.0	1.3
Glutathione S-transferase omega-1	GSTO1_HUMAN	P78417	28 kDa	0.3	0.7
Isoform 3 of Hepatoma-derived growth factor	HDGF_HUMAN	P51858-3	28 kDa	0.3	0.3
Isoform 2 of Clusterin	CLUS_HUMAN	P10909-2	58 kDa	2.3	0.3
Cysteine and glycine-rich protein 1	CSRP1_HUMAN	P21291	21 kDa	0.7	1.7
Isoform 2 of Drebrin	DREB_HUMAN	Q16643-2	72 kDa	2.0	0.7
Lactoylglutathione lyase	LGUL_HUM	Q04760	21	0.3	0.7

	AN		kDa		
Rab GDP dissociation inhibitor alpha	GDIA_HUMAN	P31150	51 kDa	1.3	3.7
Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3_HUMAN	Q06033	100 kDa	1.0	0.3
Protein S100-A4	S10A4_HUMAN	P26447	12 kDa	0.3	0.3
Protein FAM49B	FA49B_HUMAN	Q9NUQ9	38 kDa	0.3	0.3
Isoform 5 of Protein disulfide-isomerase A6	PDIA6_HUMAN	Q15084-5	53 kDa	0.7	1.3
Peroxiredoxin-1	PRDX1_HUMAN	Q06830	22 kDa	0.3	0.3
Fumarylacetoacetase	FAAA_HUMAN	P16930	46 kDa	0.0	0.3
Peroxiredoxin-5, mitochondrial	PRDX5_HUMAN	P30044	22 kDa	0.3	0.7
Isoform 1B of Desmocollin-1	DSC1_HUMAN	Q08554-2	94 kDa	0.3	0.3
High mobility group protein B2	HMGB2_HUMAN	P26583	24 kDa	0.3	0.7
Nucleosome assembly protein 1-like 1	F5H4R6_HUMAN	F5H4R6	45 kDa	2.0	1.7
Heme-binding protein 2	HEBP2_HUMAN	Q9Y5Z4	23 kDa	0.7	0.7
Prothymosin alpha	Q15203_HUMAN	Q15203	8 kDa	1.0	0.3
Cystatin-C	CYTC_HUMAN	P01034	16 kDa	0.7	0.3
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	LHPP_HUMAN	Q9H008	29 kDa	0.7	0.0
Isoform 2 of Calnexin	CALX_HUMAN	P27824-2	72 kDa	1.0	0.3
Heterochromatin protein 1-binding protein 3	HP1B3_HUMAN	Q5SSJ5	61 kDa	0.7	0.3
UV excision repair protein RAD23 homolog B	RD23B_HUMAN	P54727	43 kDa	1.0	0.0
Neutrophil defensin 3	DEF3_HUM	P59666	10	0.0	2.0

	AN		kDa		
Eosinophil cationic protein	ECP_HUMAN	P12724	18 kDa	0.0	1.0
Cathepsin Z	CATZ_HUMAN	Q9UBR2	34 kDa	1.0	0.3
Alpha-1-antitrypsin	A1AT_HUMAN	P01009	47 kDa	0.3	0.3
Elongation factor 1-beta	EF1B_HUMAN	P24534	25 kDa	1.0	0.7
Ferritin light chain	FRIL_HUMAN	P02792	20 kDa	2.0	0.7
Transforming growth factor beta-1	TGFB1_HUMAN	P01137	44 kDa	0.7	1.0
Isoform 2 of Proteasome subunit alpha type-3	PSA3_HUMAN	P25788-2	28 kDa	0.0	0.7
Peptidyl-prolyl cis-trans isomerase FKBP1A	FKB1A_HUMAN	P62942	12 kDa	0.0	0.7
Poly(rC)-binding protein 1	PCBP1_HUMAN	Q15365	37 kDa	0.0	0.7
Minor histocompatibility protein HA-1	HMHA1_HUMAN	Q92619	125 kDa	0.7	1.0
Tartrate-resistant acid phosphatase type 5	PPA5_HUMAN	P13686	37 kDa	0.3	1.0
Isoform 2 of Beta-parvin	PARVB_HUMAN	Q9HBI1-2	45 kDa	1.3	0.7
cDNA FLJ77421, highly similar to Homo sapiens autoantigen p542 mRNA	A8K4T9_HUMAN	A8K4T9	30 kDa	0.3	0.3
Apolipoprotein E	APOE_HUMAN	P02649	36 kDa	0.7	1.0
Glia maturation factor gamma	GMFG_HUMAN	O60234	17 kDa	0.0	1.0
Neutrophil collagenase	MMP8_HUMAN	P22894	53 kDa	0.0	0.7
Coronin	B4E3S0_HUMAN	B4E3S0	42 kDa	0.0	0.7
Peptidoglycan recognition protein 1	PGRP1_HUMAN	O75594	22 kDa	0.3	0.3
N-acetyl-D-glucosamine kinase	NAGK_HU	Q9UJ70	37	0.3	0.3

	MAN		kDa		
Endonuclease domain-containing 1 protein	ENDD1_HUMAN	O94919	55 kDa	0.3	1.0
Lactoferrin	W8QEY1_HUMAN	W8QEY1	78 kDa	0.0	11.3
LIM and senescent cell antigen-like-containing domain protein 1	LIMS1_HUMAN	P48059	37 kDa	0.0	1.0

Chapter 5: Summary and Future Directions

Summary

The studies presented in this dissertation provide new insight into differences in the paracrine actions of CACs as a function of habitual physical activity and CVD. Collectively, the two studies presented demonstrate that both physical inactivity and CVD alter the paracrine actions of CD34-/CD31+ CACs which in turn impair HUVEC tube formation. The first study presented identifies, for the first time, that secreted factors from CD34+ and CD34-/CD31+ CACs affect HUVEC tube formation as a function of habitual endurance-exercise with CM from inactive subjects CACs resulting in depressed tube formation compared to their active and endurance-trained counterparts. Additionally, study #1 identifies inflammatory proteins S100A8 and S100A9 as major factors contributing to the depressed tube formation observed when using CD34-/CD31+ CM inactive younger adults compared to endurance-trained athletes.

The second study aimed to confirm the effects of S100A8 and S100A9 in CD34-/CD31+ CM on HUVEC tube formation in CVD patient populations who would theoretically be candidates for autologous cell therapies using these same CACs and comparing these effects to endurance-trained athletes as a model of "optimal function". Study #2 found that the CM from NSTEMI patient CD34-/CD31+ CACs impaired tube formation compared to athletes' CM, and that pretreatment of HUVECs with an inhibitor for TLR4, a major receptor for S100A8 and S100A9, rescued tube formation to the levels observed when using CD34-/CD31+ CM from athletes. Additionally, this study found higher S100A8 and S100A9 content in the CM and higher mRNA expression of S100A8 and S100A9 in the CD34-/CD31+ CACs. Finally, the study #2 mechanistically demonstrated the direct role of S100A8 and S100A9 on tube formation when using recombinant S100A8 and S100A9 in concentrations present in NSTEMI patient CM, and confirmed that these actions were mediated by TLR4. Exploratory aims in study #2 did not indicate that CM from either group activated any of the signaling pathways downstream of TLR4. Preliminary data in study #2 suggest that cell surface markers on selected CD34-/CD31+ CACs are inherently different between NSTEMI patients and endurance-trained athletes with lower presence of T-cell and monocyte markers on the CD34-/CD31+ CACs of NSTEMI patients. These data paired with mRNA data suggest that the CD34-/CD31+ CACs from NSTEMI patients exhibit a less angiogenic phenotype compared to those from endurance-trained athletes, potentially explaining our observed differences in paracrine function.

Collectively, these studies indicate that the paracrine actions of CACs play a critical role in the cell's ability to contribute to endothelial maintenance and repair. The inhibitory effects of CM as a function of physical inactivity or CVD on HUVEC tube formation that we observed in these studies may, at least in part, explain why autologous CAC therapies in to treat ischemic CVDs produce results that are inconsistent at best. These findings are of particular importance as new methods to improve CAC function for therapeutic purposes are being developed. Methods to suppress S100A8 and S100A9 production and secretion from CD34-/CD31+ CACs of CVD patients so that they produce levels more similar to those found in endurance-trained athletes' CACs may help to improve the efficacy of these autologous treatments in the future.

Limitations and Future Directions

Although the studies presented in this dissertation provide a novel contribution to the literature regarding CAC paracrine function, there are several factors that should be considered for future investigations. One of the limitations of the final study is not including a healthy older group as a control. While the goal of our study was to compare the NSTEMI patients to what our lab has defined as a model of optimal CAC function, the inclusion of this third group would allow us to compare our findings to an age-matched control group. Study #1 demonstrates that differences in CAC paracrine function exist even in younger, healthy adults as a function of endurance exercise training habits, but the degree to which changes in CAC paracrine function exist with aging are not yet known. Kim et al. (2014) explored various characteristics of CD31+ CACs in older healthy controls compared to CAD patients and found substantial differences in a number of phenotypical and functional characteristics, although paracrine function was not assessed (76). Thus, it would be interesting to determine how a similar older healthy population would compare to our young endurance-trained population and the NSTEMI patients. Additionally, although study #2 is the first, to our knowledge, to explore paracrine functions of CACs in NSTEMI patients, other CVD patient populations that may be candidates for autologous CAC treatments should also be explored in future studies to determine whether differences exist with chronic and acute CVDs.

An exploratory aim of study #2 of this dissertation attempted to examine some of the signaling pathways within HUVECs downstream of TLR4 and RAGE in order to better understand how S100A8 and S100A9 present in the CD34-/CD31+ CM negatively influence HUVEC tube formation. Other studies have found that upon binding to their receptor on endothelial cells, S100A8 and S100A9 cause cytoskeletal reorganization and increase endothelial permeability through MAPK signaling pathways (43, 162). Wang et al. demonstrated that S100A8, S100A9, and the S100A8/A9 heterodimer complex act through RAGE and TLR4 to activate p38 and ERK1/2 signaling pathways. There were several factors that limited our ability to fully explore these signaling pathways for this exploratory aim. The volume of CM that was available to perform these experiments was low and as such we were limited in the way the experiments were conducted. For this aim, exposure of HUVECs to the CM was performed on harvested HUVECs, rather than adherent cells as had been done in other studies (162). Future studies using adherent HUVECs would allow for more appropriate analyses since signaling is likely altered in suspended cells. Additionally, there was a low protein yield from the HUVECs after treatments took place. Protein assays indicated that the total protein present was on the low end of the standard curve (~5-10 μg/mL), and total protein gels performed indicated very low total protein loading. As such, it is likely that calculations estimating the volumes of each sample to load for immunoblot experiment were inaccurate leading to skewed results when probing for our target protein and high coefficients of variation for each target. Thus, an important future direction would be to further optimize the methods to best test this aim and to follow up on this research question.

Inhibitors of p38 (SB203580) and ERK1/2 (PD98059) are also available (Sigma) and have been successfully used to study signaling mechanisms in HUVECs in other studies (162). Thus, there is potential for further experimentation with these signaling pathways in the future to gain more insight into the signaling mechanisms that are affecting HUVECs as a result of CM from CVD patients and endurance-trained subjects.

In addition to the intracellular signaling pathways of HUVECs upon exposure to CM from CD34-/CD31+ CACs, future studies should also focus on determining intracellular CAC functions that are potentially contributing to the increased production and secretion of S100A8 and S100A9 in NSTEMI patients compared to healthy controls. Study #1 of this dissertation investigated the role of intracellular ROS and NO as a function of habitual physical activity that might explain the differential paracrine effects. However, we found no differences between groups in either ROS or NO. Measurement of ROS and NO can be difficult due to a short half-life and a lack of globally accepted methods available to assess these functions. Thus, identification of better methods to assess these and other indicators of intracellular CAC signaling will help to better address this question. More information regarding intracellular function of CACs that are affecting paracrine actions will potentially allow for the development of new methods that can manipulate the production and secretion of particular factors to enhance the ability of CACs from CVD patients to be used for autologous therapies.

In study #2 of this dissertation, we generated preliminary data using n=3/group to assess potential differences in cell surface markers that had been previously been shown to be differentially expressed in selected CD31+ CACs as a function of CVD (76). Although the sample size used for the analyses is study #2 was not large enough to detect significant differences, we did find that athletes CD34-/CD31+ CAC expressed nearly twice as much CD3 and CD14 compared to the NSTEMI cells. These preliminary findings suggest that despite selecting for CD34-/CD31+ in both groups, the selected cells are still inherently different with the presence of CVD and warrants follow-up work. Future studies should aim at further identifying potential differences in these cell surface markers using a larger sample size as well as identifying other potentially different surface markers that may give further indication as to how these cells are functioning.

The two studies presented in this dissertation employed the HUVEC-based tube formation assay to assess angiogenesis. This *in vitro* assay provides a global readout of the entire angiogenesis cascade but does not individually assess all the critical regulatory steps during angiogenesis. This assay was selected due to the ability to perform it on each individual day using fresh CM, since preliminary experiments using frozen CM indicate that there is degradation of many proteins and outcomes have not been successful thus far. Future studies should include measures such as HUVEC proliferation and migration as a complement to the angiogenesis assay to more comprehensively assess angiogenic activity in endothelial cells. Additionally, other assays that would allow for assessment of the effects of CM or S100A8 and S100A9 on endothelial function should also be considered for future investigations.

Generation of CM and preparation of CM samples between study #1 and study #2 were further optimized such that we identified more than 250 proteins in the CD34-/CD31+ CM in study #2. The focus of this dissertation work was on just S100A8 and S100A9 and although we have empirically shown that these proteins are significantly influencing HUVEC tube formation, we can not exclude the likelihood that other proteins present in the CM are both differentially expressed and contributing to the observed differences in tube formation as a function of physical inactivity and CVD. Indeed, proteomics results from study #2 investigating n=3 subjects per group indicate that there are multiple protein targets that are several fold different in the NSTEMIs compared to the athletes. Future investigations into these specific targets and the role(s) they play in the regulation of angiogenic processes as well as the potential interplay between these proteins and S100A8/A9 would provide a more detailed picture as to how CD34-/CD31+ CAC paracrine function works. Additionally, due to our findings in study #1 indicating that S100A8 and S100A9 content in the CM of CD34-/CD31+ CACs was differentially expressed as a function of chronic endurance exercise habits, we did not follow up on the CD34+ CACs. However, we do have preliminary proteomics data on CM from both CD34+ and CD34-/CD31- CACs of NSTEMIs and endurance-trained athletes that can be used for future investigations.

The likelihood that there are factors present in the CM aside from proteins that are potentially affecting tube formation should not be overlooked. Sahoo et al., found that exosomes were responsible for the paracrine effects of isolated CD34+ cells on angiogenesis and CM depleted of exosomes failed to create tube-like formations *in vitro*. Additionally, they found that microRNA-126 and microRNA-130a had known roles in promoting angiogenesis and were found in high concentrations in CD34+ CAC exosomes compared to total mononuclear cells (125). Exosomes are secreted bodies containing cytokines, mRNAs, microRNAs and other small particles. It is believed that exosomes help maintain stability of these factors as they are transported to and act on other cells. It is possible that some other factors being transported in exosomes, such as other proteins or microRNAs are being secreted in a differential manner or that the exosomes secreted by CVD patients' CACs are dysfunctional causing early degradation of paracrine factors or inability of the exosomes to become internalized and act on HUVEC cells. We have a current collaboration with another lab in which they are quantifying the number and size of extracellular vesicles present in the CM of CD34-/CD31+ CACs. Additionally, these extracellular vesicles have been isolated and we will be comparing the expression of 84 CVD- related miRNA targets (Human Cardiovascular Disease miRNA PCRArray, Qiagen) in the CM of both athlete and NSTEMI patients' CD34-/CD31+ CACs. These data will provide us with a number of potential avenues to follow up on to better ascertain the total effects of paracrine factors released by CD34-/CD31+ CACs and how they contribute to angiogenesis. Additionally, as we continue to identify paracrine factors that could be used for therapeutic purposes, it is our hope that this collaboration will help mold further investigations into the use of extracellular vesicles from CACs as a method for delivery of specific factors to promote repair of ischemic tissue.

Together, the findings presented in this dissertation work, in addition to future directions presented above provide new understandings into the paracrine role of CACs and modulation of these actions by physical inactivity and CVD. Examination of the mechanisms affecting the paracrine function of CACs from individuals from different backgrounds is critical in order to better optimize the use of autologous CAC therapy to treat ischemic CVDs.

Literature Cited

1. **Aicher A**, **Heeschen C**, **Mildner-Rihm C**, **Urbich C**, **Ihling C**, **Technau-Ihling K**, **Zeiher AM**, **Dimmeler S**. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 9: 1370–1376, 2003.

2. **Arnaoutova I**, **George J**, **Kleinman HK**, **Benton G**. The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis* 12: 267–274, 2009.

3. **Asahara T**, **Kawamoto A**, **Masuda H**. Concise Review: Circulating Endothelial Progenitor Cells for Vascular Medicine. *Stem Cells* 29: 1650–1655, 2011.

4. **Asahara T**. Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. *Science* 275: 964–966, 1997.

5. **Averill MM**, **Kerkhoff C**, **Bornfeldt KE**. S100A8 and S100A9 in Cardiovascular Biology and Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology* 32: 223–229, 2012.

6. **Awad O**. Differential Healing Activities of CD34+ and CD14+ Endothelial Cell Progenitors. *Arteriosclerosis, Thrombosis, and Vascular Biology* 26: 758–764, 2006.

7. **Bakogiannis C**, **Tousoulis D**, **Androulakis E**, **Briasoulis A**, **Papageorgiou N**, **Vogiatzi G**, **Kampoli A-M**, **Charakida M**, **Siasos G**, **Latsios G**, **Antoniades C**, **Stefanadis C**. Circulating endothelial progenitor cells as biomarkers for prediction of cardiovascular outcomes. *Curr Med Chem* 19: 2597–2604, 2012.

8. **Bielak LF**, **Horenstein RB**, **Ryan KA**, **Sheedy PF**, **Rumberger JA**, **Tanner K**, **Post W**, **Mitchell BD**, **Shuldiner AR**, **Peyser PA**. Circulating CD34+ Cell Count is Associated with Extent of Subclinical Atherosclerosis in Asymptomatic Amish Men, Independent of 10-Year Framingham Risk. *Clin Med Cardiol* 3: 53–60, 2009.

9. **Boizeau ML**, **Fons P**, **Cousseins L**, **Desjobert J**, **Sibrac D**, **Michaux C**, **Nestor AL**, **Gautret B**, **Neil K**, **Herbert C**, **Herault JP**, **Talbot H**, **Bono F**. Automated Image Analysis of In Vitro Angiogenesis Assay. *Journal of Laboratory Automation* 18: 411–415, 2013.

10. **Bonsignore MR**, **Morici G**, **Riccioni R**, **Huertas A**, **Petrucci E**, **Veca M**, **Mariani G**, **Bonanno A**, **Chimenti L**, **Gioia M**, **Palange P**, **Testa U**. Hemopoietic and angiogenetic progenitors in healthy athletes: different responses to endurance and maximal exercise. *Journal of Applied Physiology* 109: 60–67, 2010.

11. **Boyd JH**, **Kan B**, **Roberts H**, **Wang Y**, **Walley KR**. S100A8 and S100A9 Mediate Endotoxin-Induced Cardiomyocyte Dysfunction via the Receptor for Advanced Glycation End Products. *Circulation Research* 102: 1239–1246, 2008.

12. **Braunwald E**. Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. *N Engl J Med* 337: 1360–1369, 1997.

13. **Brunt KR**, **Wu J**, **Chen Z**, **Poeckel D**, **Dercho RA**, **Melo LG**, **Funk CD**, **Ward CA**, **Li R-K**. Ex Vivo Akt/HO-1 Gene Therapy to Human Endothelial Progenitor Cells Enhances Myocardial Infarction Recovery. *cell transplant* 21: 1443–1461, 2012.

14. **Cacciatore F**, **Bruzzese G**, **Vitale DF**, **Liguori A**, **de Nigris F**, **Fiorito C**, **Infante T**, **Donatelli F**, **Minucci PB**, **Ignarro LJ**, **Napoli C**. Effects of ACE inhibition on circulating endothelial progenitor cells, vascular damage, and oxidative stress in hypertensive patients. *Eur J Clin Pharmacol* 67: 877–883, 2011.

15. **Caligiuri G**. Reduced Immunoregulatory CD31+ T Cells in Patients With Atherosclerotic Abdominal Aortic Aneurysm. *Arteriosclerosis, Thrombosis, and Vascular Biology* 26: 618–623, 2005.

16. **Cheng S**, **Wang N**, **Larson MG**, **Palmisano JN**, **Mitchell GF**, **Benjamin EJ**, **Vasan RS**, **Levy D**, **McCabe EL**, **Vita JA**, **Wang TJ**, **Shaw SY**, **Cohen KS**, **Hamburg NM**. Atherosclerosis. *Atherosclerosis* 220: 145–150, 2012.

17. **Cotoi OS**, **Dunér P**, **Ko N**, **Hedblad B**, **Nilsson J**, **Björkbacka H**, **Schiopu A**. Plasma S100A8/A9 Correlates With Blood Neutrophil Counts, Traditional Risk Factors, and Cardiovascular Disease in Middle-Aged Healthy Individuals. *Arteriosclerosis, Thrombosis, and Vascular Biology* 34: 202–210, 2014.

18. **Croce K**, **Gao H**, **Wang Y**, **Mooroka T**, **Sakuma M**, **Shi C**, **Sukhova GK**, **Packard RRS**, **Hogg N**, **Libby P**, **Simon DI**. Myeloid-Related Protein-8/14 Is Critical for the Biological Response to Vascular Injury. *Circulation* 120: 427–436, 2009.

19. **Csiszar A**, **Sosnowska D**, **Tucsek Z**, **Gautam T**, **Toth P**, **Losonczy G**, **Colman RJ**, **Weindruch R**, **Anderson RM**, **Sonntag WE**, **Ungvari Z**. Circulating Factors Induced by Caloric Restriction in the Nonhuman Primate Macaca Mulatta Activate Angiogenic Processes in Endothelial Cells. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 68: 235–249, 2013.

20. **Cui X**, **Zhang X**, **Guan X**, **Li H**, **Li X**, **Lu H**, **Cheng M**. Shear stress augments the endothelial cell differentiation marker expression in late EPCs by upregulating integrins. *Biochem Biophys Res Commun* 425: 419–425, 2012.

21. **Daub K**, **Langer H**, **Seizer P**, **Stellos K**, **May AE**, **Goyal P**, **Bigalke B**, **Schonberger T**, **Geisler T**, **Siegel-Axel D**, **Oostendorp RAJ**, **Lindemann S**, **Gawaz M**. Platelets induce differentiation of human CD34+ progenitor cells into foam cells and endothelial cells. *FASEB J* 20: 2559–2561, 2006.

22. **DeVan AE**, **Seals DR**. Vascular health in the ageing athlete. *Experimental Physiology* (January 2012). doi: 10.1113/expphysiol.2011.058792.

23. **Dimmeler S**. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *Journal of Clinical Investigation* 108: 391–397, 2001.

24. **Domanchuk K**, **Ferrucci L**, **Guralnik JM**, **Criqui MH**, **Tian L**, **Liu K**, **Losordo D**, **Stein J**, **Green D**, **Kibbe M**, **Zhao L**, **Annex B**, **Perlman H**, **Lloyd-Jones D**, **Pearce W**, **Taylor D**, **McDermott MM**. Contemporary Clinical Trials. *Contemporary Clinical Trials* 36: 502–509, 2013.

25. **Ehlermann P**, **Eggers K**, **Bierhaus A**, **Most P**, **Weichenhan D**, **Greten J**, **Nawroth PP**, **Katus HA**, **Remppis A**. *Cardiovasc Diabetol* 5: 6, 2006.

26. **Ehrchen JM**, **Sunderkötter C**, **Foell D**, **Vogl T**, **Roth J**. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol* 86: 557–566, 2009.

27. **Eue I**, **König S**, **Pior J**, **Sorg C**. S100A8, S100A9 and the S100A8/A9 heterodimer complex specifically bind to human endothelial cells: identification and characterization of ligands for the myeloid-related proteins S100A9 and S100A8/A9 on human dermal microvascular endothelial cell line-1 cells. *Int Immunol* 14: 287–297, 2002.

28. **Everaert BR**, **Van Craenenbroeck EM**, **Hoymans VY**, **Haine SE**, **Van Nassauw L**, **Conraads VM**, **Timmermans J-P**, **Vrints CJ**. International Journal of Cardiology. *International Journal of Cardiology* 144: 350–366, 2010.

29. **Fadini GP**, **Losordo D**, **Dimmeler S**. Critical Reevaluation of Endothelial Progenitor Cell Phenotypes for Therapeutic and Diagnostic Use. *Circulation Research* 110: 624–637, 2012.

30. **Fagerhol MK**, **Nielsen HG**, **Vetlesen A**, **Sandvik K**, **Lyberg T**. Increase in plasma calprotectin during long-distance running. *Scand J Clin Lab Invest* 65: 211–220, 2005.

31. **Falkenberg H**, **Radke TF**, **Kögler G**, **Stühler K**. Proteomic Profiling of Ex Vivo Expanded CD34-Positive Haematopoetic Cells Derived from Umbilical Cord Blood. *Stem Cells International* 2013: 1–9, 2013.

32. **Favre J**, **Terborg N**, **Horrevoets AJG**. The diverse identity of angiogenic monocytes. *European Journal of Clinical Investigation* 43: 100–107, 2012.

33. **Fernandes T**, **Nakamuta JS**, **Magalhães FC**, **Roque FR**, **Lavini-Ramos C**, **Schettert IT**, **Coelho V**, **Krieger JE**, **Oliveira EM**. Exercise training restores the endothelial progenitor cells number and function in hypertension: implications for angiogenesis. *J Hypertens* 30: 2133–2143, 2012.

34. **Flego D**, **Severino A**, **Trotta F**, **Previtero M**, **Ucci S**, **Zara C**, **Pedicino D**, **Massaro G**, **Biasucci LM**, **Liuzzo G**, **Crea F**. Altered CD31 expression and activity in helper T cells of acute coronary syndrome patients. *Basic Res Cardiol* 109: 448, 2014.

35. **Fleissner F**, **Thum T**. Critical role of the nitric oxide/reactive oxygen species balance in endothelial progenitor dysfunction. *Antioxid Redox Signal* 15: 933–948, 2011.

36. **Frosch M**, **Strey A**, **Vogl T**, **Wulffraat NM**, **Kuis W**, **Sunderkötter C**, **Harms E**, **Sorg C**, **Roth J**. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 43: 628–637, 2000.

37. **Fujita Y**, **Kinoshita M**, **Furukawa Y**, **Nagano T**, **Hashimoto H**, **Hirami Y**, **Kurimoto Y**, **Arakawa K**, **Yamazaki K**, **Okada Y**, **Katakami N**, **Uno E**, **Matsubara Y**, **Fukushima M**, **Nada A**, **Losordo DW**, **Asahara T**, **Okita Y**, **Kawamoto A**. Phase II Clinical Trial of CD34+ Cell Therapy to Explore Endpoint Selection and Timing in Patients With Critical Limb Ischemia. *Circ J* (2013). doi: 10.1253/circj.CJ-13-0864.

38. **Gnecchi M**, **Zhang Z**, **Ni A**, **Dzau VJ**. Paracrine Mechanisms in Adult Stem Cell Signaling and Therapy. *Circulation Research* 103: 1204–1219, 2008.

39. **Groleau J**, **Dussault S**, **Haddad P**, **Turgeon J**, **Menard C**, **Chan JS**, **Rivard A**. Essential Role of Copper-Zinc Superoxide Dismutase for Ischemia-Induced Neovascularization Via Modulation of Bone Marrow-Derived Endothelial Progenitor Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* 30: 2173–2181, 2010.

40. **Guhanarayan G**, **Jablonski J**, **Witkowski S**. Circulating angiogenic cell population responses to 10 days of reduced physical activity. *Journal of Applied Physiology* 117: 500–506, 2014.

41. **Harraz M**, **Jiao C**, **Hanlon HD**, **Hartley RS**, **Schatteman GC**. CD34- blood-derived human endothelial cell progenitors. *Stem Cells* 19: 304–312, 2001.

42. **Harry BL**, **Sanders JM**, **Feaver RE**, **Lansey M**, **Deem TL**, **Zarbock A**, **Bruce AC**, **Pryor AW**, **Gelfand BD**, **Blackman BR**, **Schwartz MA**, **Ley K**. Endothelial Cell PECAM-1 Promotes Atherosclerotic Lesions in Areas of Disturbed Flow in ApoE-Deficient Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* 28: 2003–2008, 2008.

43. **Healy AM**. Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events. *Circulation* 113: 2278–2284, 2006.

44. **HEESCHEN C**. Profoundly Reduced Neovascularization Capacity of Bone Marrow Mononuclear Cells Derived From Patients With Chronic Ischemic Heart Disease. *Circulation* 109: 1615–1622, 2004.

45. **Heidenreich PA**, **Trogdon JG**, **Khavjou OA**, **Butler J**, **Dracup K**, **Ezekowitz MD**, **Finkelstein EA**, **Hong Y**, **Johnston SC**, **Khera A**, **Lloyd-Jones DM**, **Nelson SA**, **Nichol G**, **Orenstein D**, **Wilson PWF**, **Woo YJ**, **on behalf of the American Heart Association Advocacy Coordinating Committee**, **Stroke Council**, **Council on Cardiovascular Radiology and Intervention**, **Council on Clinical Cardiology**, **Council on Epidemiology and Prevention**, **Council on Arteriosclerosis**, **Thrombosis and Vascular Biology**, **Council on Cardiopulmonary**, **Critical Care**, **Perioperative and Resuscitation**, **Council on Cardiovascular Nursing**, **Council on the Kidney in Cardiovascular Disease**, **Council on Cardiovascular Surgery and Anesthesia, and Interdisciplinary Council on Quality of Care and Outcomes Research**. Forecasting the Future of Cardiovascular Disease in the United States: A Policy Statement From the American Heart Association. *Circulation* 123: 933–944, 2011.

46. **Hill JM**, **Zalos G**, **Halcox JPJ**, **Schenke WH**, **Waclawiw MA**, **Quyyumi AA**, **Finkel T**. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348: 593–600, 2003.

47. **Hill JM**, **Zalos G**, **Halcox JPJ**, **Schenke WH**, **Waclawiw MA**, **Quyyumi AA**, **Finkel T**. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348: 593–600, 2003.

48. **Hobbs JAR**, **May R**, **Tanousis K**, **McNeill E**, **Mathies M**, **Gebhardt C**, **Henderson R**, **Robinson MJ**, **Hogg N**. Myeloid Cell Function in MRP-14 (S100A9) Null Mice. *Molecular and Cellular Biology* 23: 2564–2576, 2003.

49. **Hoetzenecker K**, **Zimmermann M**, **Hoetzenecker W**, **Schweiger T**, **Kollmann D**, **Mildner M**, **Hegedus B**, **Mitterbauer A**, **Hacker S**, **Birner P**, **Gabriel C**, **Gyongyosi M**, **Blyszczuk P**, **Eriksson U**, **Ankersmit HJ**. Mononuclear cell secretome protects from experimental autoimmune myocarditis. *European Heart Journal* 36: 676–685, 2015.

50. **Hoetzer GL**, **Van Guilder GP**, **Irmiger HM**, **Keith RS**, **Stauffer BL**, **DeSouza CA**. Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men. *J Appl Physiol* 102: 847–852, 2007.

51. **Horrevoets AJG**. Angiogenic Monocytes. *Am J Pathol* 174: 1594–1596, 2009.

52. **Hur J**, **Yang HM**, **Yoon CH**, **Lee CS**, **Park KW**, **Kim JH**, **Kim TY**, **Kim JY**,

Kang HJ, **Chae IH**, **Oh BH**, **Park YB**, **Kim HS**. Identification of a Novel Role of T Cells in Postnatal Vasculogenesis: Characterization of Endothelial Progenitor Cell Colonies. *Circulation* 116: 1671–1682, 2007.

53. **Hur J**. Characterization of Two Types of Endothelial Progenitor Cells and Their Different Contributions to Neovasculogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology* 24: 288–293, 2003.

54. **Hynes B**, **Kumar AHS**, **O'Sullivan J**, **Klein Buneker C**, **Leblond A-L**, **Weiss S**, **Schmeckpeper J**, **Martin K**, **Caplice NM**. Potent endothelial progenitor cell-conditioned media-related anti-apoptotic, cardiotrophic, and pro-angiogenic effects post-myocardial infarction are mediated by insulin-like growth factor-1. *European Heart Journal* 34: 782–789, 2011.

55. **Ii M**. A Novel Cyclohexene Derivative, Ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), Selectively Inhibits Toll-Like Receptor 4-Mediated Cytokine Production through Suppression of Intracellular Signaling. *Molecular Pharmacology* 69: 1288–1295, 2006.

56. **Imanishi T**, **Hano T**, **Sawamura T**, **Nishio I**. Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clin Exp Pharmacol Physiol* 31: 407–413, 2004.

57. **Ingram DA**, **Krier TR**, **Mead LE**, **McGuire C**, **Prater DN**, **Bhavsar J**, **Saadatzadeh MR**, **Bijangi-Vishehsaraei K**, **Li F**, **Yoder MC**, **Haneline LS**. Clonogenic Endothelial Progenitor Cells Are Sensitive to Oxidative Stress. *Stem Cells* 25: 297–304, 2007.

58. **Iwaguro H**. Endothelial Progenitor Cell Vascular Endothelial Growth Factor Gene Transfer for Vascular Regeneration. *Circulation* 105: 732–738, 2001.

59. **Iwakura A**. Estrogen-Mediated, Endothelial Nitric Oxide Synthase-Dependent Mobilization of Bone Marrow-Derived Endothelial Progenitor Cells Contributes to Reendothelialization After Arterial Injury. *Circulation* 108: 3115–3121, 2003.

60. **Iwakura A**. Estradiol Enhances Recovery After Myocardial Infarction by Augmenting Incorporation of Bone Marrow-Derived Endothelial Progenitor Cells Into Sites of Ischemia-Induced Neovascularization via Endothelial Nitric Oxide Synthase-Mediated Activation of Matrix Metalloproteinase-9. *Circulation* 113: 1605–1614, 2006.

61. **Iwasaki H**. Dose-Dependent Contribution of CD34-Positive Cell Transplantation to Concurrent Vasculogenesis and Cardiomyogenesis for Functional Regenerative Recovery After Myocardial Infarction. *Circulation* 113: 1311–1325, 2006.

62. **Jackson AS**, **Pollock ML**. *Generalized equations for predicting body density of*

men. 1978. 2004.

63. **Jenkins NT**, **Landers RQ**, **Prior SJ**, **Soni N**, **Spangenburg EE**, **Hagberg JM**. Effects of acute and chronic endurance exercise on intracellular nitric oxide and superoxide in circulating CD34+ and CD34- cells. *Journal of Applied Physiology* 111: 929–937, 2011.

64. **Jenkins NT**, **Landers RQ**, **Thakkar SR**, **Fan X**, **Brown MD**, **Prior SJ**, **Spangenburg EE**, **Hagberg JM**. Prior endurance exercise prevents postprandial lipaemia-induced increases in reactive oxygen species in circulating CD31+ cells. *J Physiol (Lond)* 589: 5539–5553, 2011.

65. **Jenkins NT**, **Martin JS**, **Laughlin MH**, **Padilla J**. Exercise-Induced Signals for Vascular Endothelial Adaptations: Implications for Cardiovascular Disease. *Curr Cardiovasc Risk Rep* 6: 331–346, 2012.

66. **Jenkins NT**, **Witkowski S**, **Spangenburg EE**, **Hagberg JM**. Effects of acute and chronic endurance exercise on intracellular nitric oxide in putative endothelial progenitor cells: role of NAPDH oxidase. *AJP: Heart and Circulatory Physiology* 297: H1798–H1805, 2009.

67. **Jiraritthamrong C**, **Kheolamai P**, **U-Pratya Y**, **Chayosumrit M**, **Supokawej A**, **Manochantr S**, **Tantrawatpan C**, **Sritanaudomchai H**, **Issaragrisil S**. In vitro vessel-forming capacity of endothelial progenitor cells in high glucose conditions. *Ann Hematol* 91: 311–320, 2011.

68. **Kakizaki M**, **Nobori K**, **Watanabe H**, **Iino K**, **Ishida M**, **Ito H**. Increased circulating CD3+/CD31+ T cells in patients with acute coronary syndrome. *Heart Vessels* 28: 566–569, 2012.

69. **Kalka C**, **Masuda H**, **Takahashi T**, **Kalka-Moll WM**, **Silver M**, **Kearney M**, **Li T**, **Isner JM**, **Asahara T**. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA* 97: 3422–3427, 2000.

70. **Kang S**, **Park HS**, **Jo A**, **Hong SH**, **Lee HN**, **Lee YY**, **Park JS**, **Jung HS**, **Chung SS**, **Park KS**. Endothelial progenitor cell cotransplantation enhances islet engraftment by rapid revascularization. *Diabetes* 61: 866–876, 2012.

71. **Katashima T**, **Naruko T**, **Terasaki F**, **Fujita M**, **Otsuka K**, **Murakami S**, **Sato A**, **Hiroe M**, **Ikura Y**, **Ueda M**, **Ikemoto M**, **Kitaura Y**. Enhanced Expression of the S100A8/A9 Complex in Acute Myocardial Infarction Patients. *Circ J* 74: 741–748, 2010.

72. **Kawamoto A**, **Iwasaki H**, **Kusano K**, **Murayama T**, **Oyamada A**, **Silver M**, **Hulbert C**, **Gavin M**, **Hanley A**, **Ma H**, **Kearney M**, **Zak V**, **Asahara T**, **Losordo DW**. CD34-Positive Cells Exhibit Increased Potency and Safety for Therapeutic Neovascularization After Myocardial Infarction Compared With

Total Mononuclear Cells. *Circulation* 114: 2163–2169, 2006.

73. **Kawamoto T**, **Ii M**, **Kitazaki T**, **Iizawa Y**, **Kimura H**. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *European Journal of Pharmacology* 584: 40–48, 2008.

74. **Kerkhoff C**, **Klempt M**, **Sorg C**. Novel insights into structure and function of MRP8 (S100A8) and MRP14 (S100A9). *Biochim Biophys Acta* 1448: 200–211, 1998.

75. **Kim H**, **Cho HJ**, **Kim SW**, **Liu B**, **Choi YJ**, **Lee J**, **Sohn YD**, **Lee MY**, **Houge MA**, **Yoon YS**. CD31+ Cells Represent Highly Angiogenic and Vasculogenic Cells in Bone Marrow: Novel Role of Nonendothelial CD31+ Cells in Neovascularization and Their Therapeutic Effects on Ischemic Vascular Disease. *Circulation Research* 107: 602–614, 2010.

76. **Kim MH**, **Guo L**, **Kim H-S**, **Kim S-W**. Characteristics of circulating CD31 +cells from patients with coronary artery disease. *J Cell Mol Med* 18: 2321–2330, 2014.

77. **Kim S-J**, **Kim J-S**, **Papadopoulos J**, **Wook Kim S**, **Maya M**, **Zhang F**, **He J**, **Fan D**, **Langley R**, **Fidler IJ**. Circulating Monocytes Expressing CD31. *Am J Pathol* 174: 1972–1980, 2009.

78. **Kim S-W**, **Kim H**, **Yoon Y-S**. Advances in bone marrow-derived cell therapy: CD31-expressing cells as next generation cardiovascular cell therapy. *Regenerative Medicine* 6: 335–349, 2011.

79. **Kim SW**, **Kim H**, **Cho HJ**, **Lee JU**, **Levit R**, **Yoon Y**. Human Peripheral Blood-Derived CD31+ Cells Have Robust Angiogenic and Vasculogenic Properties and Are Effective for Treating Ischemic Vascular Disease. *JAC* 56: 593–607, 2010.

80. **Kinnaird T**. Marrow-Derived Stromal Cells Express Genes Encoding a Broad Spectrum of Arteriogenic Cytokines and Promote In Vitro and In Vivo Arteriogenesis Through Paracrine Mechanisms. *Circulation Research* 94: 678–685, 2004.

81. **Kinnaird T**. Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms. *Circulation* 109: 1543–1549, 2004.

82. **Koutroumpi M**. Circulating endothelial and progenitor cells: Evidence from acute and long-term exercise effects. *WJC* 4: 312, 2012.

83. **Kruger K**, **Klocke R**, **Kloster J**, **Nikol S**, **Waltenberger J**, **Mooren FC**. Activity of daily living is associated with circulating CD34+/KDR+ cells and granulocyte colony-stimulating factor levels in patients after myocardial

infarction. *Journal of Applied Physiology* 116: 532–537, 2014.

84. **Kushner EJ**, **MacEneaney OJ**, **Morgan RG**, **Van Engelenburg AM**, **Van Guilder GP**, **DeSouza CA**. Blood Cells, Molecules, and Diseases. *Blood Cells, Molecules, and Diseases* 44: 74–78, 2010.

85. **Kushner EJ**, **MacEneaney OJ**, **Weil BR**, **Greiner JJ**, **Stauffer BL**, **DeSouza CA**. Aging Is Associated with a Proapoptotic Endothelial Progenitor Cell Phenotype. *J Vasc Res* 48: 408–414, 2011.

86. **Kushner EJ**, **Weil BR**, **MacEneaney OJ**, **Morgan RG**, **Mestek ML**, **Van Guilder GP**, **Diehl KJ**, **Stauffer BL**, **DeSouza CA**. Human aging and CD31+ T-cell number, migration, apoptotic susceptibility, and telomere length. *Journal of Applied Physiology* 109: 1756–1761, 2010.

87. **Landers-Ramos RQ**, **Sapp RM**, **Jenkins NT**, **Murphy AE**, **Cancre L**, **Chin ER**, **Spangenburg EE**, **Hagberg JM**. Chronic endurance exercise affects paracrine action of CD31+ and CD34+ cells on endothelial tube formation. *AJP: Heart and Circulatory Physiology* (June 8, 2015). doi: 10.1152/ajpheart.00123.2015.

88. **Landers-Ramos RQ**, **Sapp RM**, **Jenkins NT**, **Murphy AE**, **Cancre L**, **Chin ER**, **Spangenburg EE**, **Hagberg JM**. Chronic endurance exercise affects paracrine action of CD31 +and CD34 +cells on endothelial tube formation. *AJP: Heart and Circulatory Physiology* 309: H407–H420, 2015.

89. **Lasala GP**, **Silva JA**, **Minguell JJ**. Therapeutic angiogenesis in patients with severe limb ischemia by transplantation of a combination stem cell product. *J Thorac Cardiovasc Surg* 144: 377–382, 2012.

90. **Leifheit-Nestler M**, **Conrad G**, **Heida NM**, **Limbourg A**, **Limbourg FP**, **Seidler T**, **Schroeter MR**, **Hasenfuss G**, **Konstantinides S**, **Schafer K**. Overexpression of Integrin 5 Enhances the Paracrine Properties of Circulating Angiogenic Cells via Src Kinase-Mediated Activation of STAT3. *Arteriosclerosis, Thrombosis, and Vascular Biology* 30: 1398–1406, 2010.

91. **Lenk K**, **Uhlemann M**, **Schuler G**, **Adams V**. Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. *Journal of Applied Physiology* 111: 321–328, 2011.

92. **Li C**, **Li S**, **Jia C**, **Yang L**, **Song Z**, **Wang Y**. Low Concentration of S100A8/9 Promotes Angiogenesis-Related Activity of Vascular Endothelial Cells: Bridges among Inflammation, Angiogenesis, and Tumorigenesis? *Mediators of Inflammation* 2012: 1–8, 2012.

93. **Libby P**, **Sukhova G**, **Lee RT**, **Galis ZS**. Cytokines regulate vascular functions related to stability of the atherosclerotic plaque. *J Cardiovasc Pharmacol* 25 Suppl 2: S9–12, 1995.

94. **Linke A**, **Erbs S**, **Hambrecht R**. Effects of exercise training upon endothelial function in patients with cardiovascular disease. *Front Biosci* 13: 424–432, 2008.

95. **Losordo DW**, **Henry TD**, **Davidson C**, **Sup Lee J**, **Costa MA**, **Bass T**, **Mendelsohn F**, **Fortuin FD**, **Pepine CJ**, **Traverse JH**, **Amrani D**, **Ewenstein BM**, **Riedel N**, **Story K**, **Barker K**, **Povsic TJ**, **Harrington RA**, **Schatz RA**, **the ACT34-CMI Investigators**. Intramyocardial, Autologous CD34+ Cell Therapy for Refractory Angina. *Circulation Research* 109: 428–436, 2011.

96. **Losordo DW**, **Kibbe MR**, **Mendelsohn F**, **Marston W**, **Driver VR**, **Sharafuddin M**, **Teodorescu V**, **Wiechmann BN**, **Thompson C**, **Kraiss L**, **Carman T**, **Dohad S**, **Huang P**, **Junge CE**, **Story K**, **Weistroffer T**, **Thorne TM**, **Millay M**, **Runyon JP**, **Schainfeld R**, **for the Autologous CD34+ Cell Therapy for Critical Limb Ischemia Investigators**. A Randomized, Controlled Pilot Study of Autologous CD34+ Cell Therapy for Critical Limb Ischemia. *Circulation: Cardiovascular Interventions* 5: 821–830, 2012.

97. **Losordo DW**, **Schatz RA**, **White CJ**, **Udelson JE**, **Veereshwarayya V**, **Durgin M**, **Poh KK**, **Weinstein R**, **Kearney M**, **Chaudhry M**, **Burg A**, **Eaton L**, **Heyd L**, **Thorne T**, **Shturman L**, **Hoffmeister P**, **Story K**, **Zak V**, **Dowling D**, **Traverse JH**, **Olson RE**, **Flanagan J**, **Sodano D**, **Murayama T**, **Kawamoto A**, **Kusano KF**, **Wollins J**, **Welt F**, **Shah P**, **Soukas P**, **Asahara T**, **Henry TD**. Intramyocardial Transplantation of Autologous CD34+ Stem Cells for Intractable Angina: A Phase I/IIa Double-Blind, Randomized Controlled Trial. *Circulation* 115: 3165–3172, 2007.

98. **Luk TH**, **Dai YL**, **Siu CW**, **Yiu KH**, **Chan HT**, **Lee SW**, **Li SW**, **Fong B**, **Wong WK**, **Tam S**, **Lau CP**, **Tse HF**. Effect of exercise training on vascular endothelial function in patients with stable coronary artery disease: a randomized controlled trial. *European Journal of Preventive Cardiology* 19: 830–839, 2012.

99. **Lundgren DH**, **Hwang S-I**, **Wu L**, **Han DK**. Role of spectral counting in quantitative proteomics. *Expert Review of Proteomics* 7: 39–53, 2010.

100. **Ma FX**. Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. *The Journal of Lipid Research* 47: 1227–1237, 2006.

101. **MacEneaney OJ**, **Kushner EJ**, **Westby CM**, **Cech JN**, **Greiner JJ**, **Stauffer BL**, **DeSouza CA**. Endothelial progenitor Cell Function,Apoptosis, and telomere Lengthin Overweight/Obese Humans. (January 6, 2010). doi: 10.1038/oby.2009.494.

102. **Mackie AR**, **Klyachko E**, **Thorne T**, **Schultz KM**, **Millay M**, **Ito A**, **Kamide CE**, **Liu T**, **Gupta R**, **Sahoo S**, **Misener S**, **Kishore R**, **Losordo DW**. Sonic Hedgehog-Modified Human CD34+ Cells Preserve Cardiac Function After Acute Myocardial Infarction. *Circulation Research* 111: 312–321, 2012.

103. **Mackie AR**, **Losordo DW**. CD34-positive stem cells: in the treatment of heart and vascular disease in human beings. *Tex Heart Inst J* 38: 474–485, 2011.

104. **Masuda H**, **Kalka C**, **Takahashi T**, **Yoshida M**, **Wada M**, **Kobori M**, **Itoh R**, **Iwaguro H**, **Eguchi M**, **Iwami Y**, **Tanaka R**, **Nakagawa Y**, **Sugimoto A**, **Ninomiya S**, **Hayashi S**, **Kato S**, **Asahara T**. Estrogen-Mediated Endothelial Progenitor Cell Biology and Kinetics For Physiological Postnatal Vasculogenesis. *Circulation Research* 101: 598–606, 2007.

105. **MBBS ASJB**, **MD GYHL**, **MD SS**, **MD ES**. The Role of Monocytes in Angiogenesis and Atherosclerosis. *JAC* 63: 1–11, 2013.

106. **MD TJP**, **PhD CJ**, **MD AN**, **MD RAS**, **MD RAH**, **MD CJD**, **MD FDF**, **MD DJK**, **MD FOM**, **MD WS**, **MD GLS**, **MD CJW**, **MD DS**, **MS KS**, **MD DWL**, **MD TDH**. A phase 3, randomized, double-blinded,active-controlled, unblinded standard of care studyassessing the efficacy and safety of intramyocardialautologous CD34+ cell administration in patients withrefractory angina: Design of the RENEW study. *American Heart Journal* 165: 854–861.e2, 2013.

107. **Medicine ACOS**. *ACSM's Guidelines for Exercise Testing and Prescription*. Eighth. Lippincott Williams & Wilkins, 2009.

108. **Medina RJ**, **O'Neill CL**, **Sweeney M**, **Guduric-Fuchs J**, **Gardiner TA**, **Simpson DA**, **Stitt AW**. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC Med Genomics* 3: 18, 2010.

109. **Mobius-Winkler S**, **Hilberg T**, **Menzel K**, **Golla E**, **Burman A**, **Schuler G**, **Adams V**. Time-dependent mobilization of circulating progenitor cells during strenuous exercise in healthy individuals. *Journal of Applied Physiology* 107: 1943–1950, 2009.

110. **Mocharla P**, **Briand S**, **Giannotti G**, **Dorries C**, **Jakob P**, **Paneni F**, **Luscher T**, **Landmesser U**. AngiomiR-126 expression and secretion from circulating CD34+ and CD14+ PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. *Blood* 121: 226–236, 2013.

111. **Mooren F**, **Lechtermann A**, **Fobker M**, **Brandt B**, **Sorg C**, **Völker K**, **Nacken W**. The Response of the Novel Pro-Inflammatory Molecules S100A8/A9 to Exercise. *Int J Sports Med* 27: 751–758, 2006.

112. **Mora S**, **Cook N**, **Buring JE**, **Ridker PM**, **Lee I-M**. Physical Activity and Reduced Risk of Cardiovascular Events: Potential Mediating Mechanisms. *Circulation* 116: 2110–2118, 2007.

113. **Mortensen OH**, **Andersen K**, **Fischer C**, **Nielsen AR**, **Nielsen S**, **Åkerström T**, **Aastrøm M-B**, **Borup R**, **Pedersen BK**. Calprotectin is released from human

skeletal muscle tissue during exercise. *J Physiol (Lond)* 586: 3551–3562, 2008.

114. **Murad S**. Toll-like receptor 4 in inflammation and angiogenesis: a double-edged sword. *Front Immunol* 5: 313, 2014.

115. **Musialek P**, **Tekieli L**, **Kostkiewicz M**, **Majka M**, **Szot W**, **Walter Z**, **Zebzda A**, **Pieniazek P**, **Kadzielski A**, **Banys RP**, **Olszowska M**, **Pasowicz M**, **Zmudka K**, **Tracz W**. Randomized transcoronary delivery of CD34+ cells with perfusion versus stop-flow method in patients with recent myocardial infarction: Early cardiac retention of 99mTc-labeled cells activity. *J Nucl Cardiol* 18: 104–116, 2010.

116. **Newton RA**, **Hogg N**. The human S100 protein MRP-14 is a novel activator of the beta 2 integrin Mac-1 on neutrophils. *J Immunol* 160: 1427–1435, 1998.

117. **Obi S**, **Masuda H**, **Shizuno T**, **Sato A**, **Yamamoto K**, **Ando J**, **Abe Y**, **Asahara T**. Fluid shear stress induces differentiation of circulating phenotype endothelial progenitor cells. *AJP: Cell Physiology* 303: C595–C606, 2012.

118. **Passey RJ**, **Xu K**, **Hume DA**, **Geczy CL**. S100A8: emerging functions and regulation. *J Leukoc Biol* 66: 549–556, 1999.

119. **Pepine CJ**. Systemic hypertension and coronary artery disease. *Am J Cardiol* 82: 21H–24H, 1998.

120. **Perin EC**, **Willerson JT**. CD34+ Autologous Human Stem Cells in Treating Refractory Angina. *Circulation Research* 109: 351–352, 2011.

121. **Poole J**, **Mavromatis K**, **Binongo JN**, **Khan A**, **Li Q**, **Khayata M**, **Rocco E**, **Topel M**, **Zhang X**, **Brown C**, **Corriere MA**, **Murrow J**, **Sher S**, **Clement S**, **Ashraf K**, **Rashed A**, **Kabbany T**, **Neuman R**, **Morris A**, **Ali A**, **Hayek S**, **Oshinski J**, **Yoon Y-S**, **Waller EK**, **Quyyumi AA**. Effect of Progenitor Cell Mobilization With Granulocyte-Macrophage Colony-Stimulating Factor in Patients With Peripheral Artery Disease. *JAMA* 310: 2631, 2013.

122. **Rakobowchuk M**, **Harris E**, **Taylor A**, **Baliga V**, **Cubbon RM**, **Rossiter HB**, **Birch KM**. Heavy and moderate interval exercise training alters low-flow-mediated constriction but does not increase circulating progenitor cells in healthy humans. *Experimental Physiology* 97: 375–385, 2012.

123. **Rammes A**, **Roth J**, **Goebeler M**, **Klempt M**, **Hartmann M**, **Sorg C**. Myeloid-related protein (MRP) 8 and MRP14, calcium-binding proteins of the S100 family, are secreted by activated monocytes via a novel, tubulin-dependent pathway. *J Biol Chem* 272: 9496–9502, 1997.

124. **Rehman J**. Peripheral Blood “Endothelial Progenitor Cells” Are Derived From Monocyte/Macrophages and Secrete Angiogenic Growth Factors. *Circulation* 107: 1164–1169, 2003.

125. **Sahoo S**, **Klychko E**, **Thorne T**, **Misener S**, **Schultz KM**, **Millay M**, **Ito A**, **Liu T**, **Kamide C**, **Agrawal H**, **Perlman H**, **Qin G**, **Kishore R**, **Losordo DW**. Exosomes From Human CD34+ Stem Cells Mediate Their Proangiogenic Paracrine Activity. *Circulation Research* 109: 724–728, 2011.

126. **Sahoo S**, **Klychko E**, **Thorne T**, **Misener S**, **Schultz KM**, **Millay M**, **Ito A**, **Liu T**, **Kamide C**, **Agrawal H**, **Perlman H**, **Qin G**, **Kishore R**, **Losordo DW**. Exosomes From Human CD34+ Stem Cells Mediate Their Proangiogenic Paracrine Activity. *Circulation Research* 109: 724–728, 2011.

127. **Salven P**. Interleukin-1a (IL-1a) promotes angiogenesis in vivo via VEGFR-2 pathway by inducing inflammatory cell VEGF synthesis and secretion. *The FASEB Journal* (July 18, 2002). doi: 10.1096/fj.02-0134fje.

128. **Schatteman GC**, **Hanlon HD**, **Jiao C**, **Dodds SG**, **Christy BA**. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest* 106: 571–578, 2000.

129. **Scheubel RJ**, **Holtz J**, **Friedrich I**, **Borgermann J**, **Kahrstedt S**, **Santos AN**, **Silber R-E**, **Simm A**. Paracrine effects of CD34 progenitor cells on angiogenic endothelial sprouting. *International Journal of Cardiology* 139: 134–141, 2010.

130. **Schiopu A**, **Cotoi OS**. S100A8 and S100A9: DAMPs at the Crossroads between Innate Immunity, Traditional Risk Factors, and Cardiovascular Disease. *Mediators of Inflammation* 2013: 1–10, 2013.

131. **Schlager O**, **Giurgea A**, **Schuhfried O**, **Seidinger D**, **Hammer A**, **Gröger M**, **Fialka-Moser V**, **Gschwandtner M**, **Koppensteiner R**, **Steiner S**. ARTICLE IN PRESS. *Atherosclerosis* (April 5, 2011). doi: 10.1016/j.atherosclerosis.2011.03.018.

132. **Schmeisser A**, **Garlichs CD**, **Zhang H**, **Eskafi S**, **Graffy C**, **Ludwig J**, **Strasser RH**, **Daniel WG**. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. *Cardiovascular Research* 49: 671–680, 2001.

133. **Seals DR**, **DeSouza CA**, **Donato AJ**, **Tanaka H**. Habitual exercise and arterial aging. *J Appl Physiol* 105: 1323–1332, 2008.

134. **Seals DR**, **Jablonski KL**, **Donato AJ**. Aging and vascular endothelial function in humans. *Clin Sci* 120: 357–375, 2011.

135. **Shaffer RG**, **Greene S**, **Arshi A**, **Supple G**, **Bantly A**, **Moores JS**, **Parmacek MS**, **Mohler ER**. Effect of acute exercise on endothelial progenitor cells in patients with peripheral arterial disease. *Vascular Medicine* 11: 219–226, 2006.

136. **Sonnenschein K**, **Horvath T**, **Mueller M**, **Markowski A**, **Siegmund T**, **Jacob C**, **Drexler H**, **Landmesser U**. Exercise training improves in vivo endothelial

repair capacity of early endothelial progenitor cells in subjects with metabolic syndrome. *European Journal of Cardiovascular Prevention & Rehabilitation* 18: 406–414, 2011.

137. **Sunahori K**, **Yamamura M**, **Yamana J**, **Takasugi K**, **Kawashima M**, **Yamamoto H**, **Chazin WJ**, **Nakatani Y**, **Yui S**, **Makino H**. *Arthritis Res Ther* 8: R69, 2006.

138. **Thijssen DHJ**, **Torella D**, **Hopman MTE**, **Ellison GM**. The role of endothelial progenitor and cardiac stem cells in the cardiovascular adaptations to age and exercise. *Front Biosci* 14: 4685–4702, 2009.

139. **Thum T**, **Fraccarollo D**, **Schultheiss M**, **Froese S**, **Galuppo P**, **Widder JD**, **Tsikas D**, **Ertl G**, **Bauersachs J**. Endothelial Nitric Oxide Synthase Uncoupling Impairs Endothelial Progenitor Cell Mobilization and Function in Diabetes. *Diabetes* 56: 666–674, 2007.

140. **Timmons BW**. Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. *Journal of Applied Physiology* 99: 979–985, 2005.

141. **Toumpanakis D**, **Karatza M-H**, **Katsaounou P**, **Roussos C**, **Zakynthinos S**, **Papapetropoulos A**, **Vassilakopoulos T**. Antioxidant supplementation alters cytokine production from monocytes. *J Interferon Cytokine Res* 29: 741–748, 2009.

142. **Tousoulis D**, **Andreou I**, **Antoniades C**, **Tentolouris C**, **Stefanadis C**. Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization: Therapeutic implications for cardiovascular diseases. *Atherosclerosis* 201: 236–247, 2008.

143. **Traverse JH**. Effect of Intracoronary Delivery of Autologous Bone Marrow Mononuclear Cells 2 to 3 Weeks Following Acute Myocardial Infarction on Left Ventricular FunctionThe LateTIME Randomized Trial. *JAMA* 306: 2110, 2011.

144. **Traverse JH**. Effect of the Use and Timing of Bone Marrow Mononuclear Cell Delivery on Left Ventricular Function After Acute Myocardial InfarctionThe TIME Randomized TrialBone Marrow Mononuclear Cell Delivery. *JAMA* (November 6, 2012). doi: 10.1001/jama.2012.28726.

145. **Urbich C**, **Aicher A**, **HEESCHEN C**, **DERNBACH E**, **HOFMANN W**, **ZEIHER A**, **Dimmeler S**. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *Journal of Molecular and Cellular Cardiology* 39: 733–742, 2005.

146. **Van Craenenbroeck EM**, **Beckers PJ**, **Possemiers NM**, **Wuyts K**, **Frederix G**, **Hoymans VY**, **Wuyts F**, **Paelinck BP**, **Vrints CJ**, **Conraads VM**. Exercise acutely reverses dysfunction of circulating angiogenic cells in chronic heart

failure. *European Heart Journal* 31: 1924–1934, 2010.

147. **Van Craenenbroeck EM**, **Conraads VM**. Endothelial progenitor cells in vascular health: Focus on lifestyle. *Microvascular Research* (January 28, 2010). doi: 10.1016/j.mvr.2009.12.009.

148. **Van Craenenbroeck EM**, **Hoymans VY**, **Beckers PJ**, **Possemiers NM**, **Wuyts K**, **Paelinck BP**, **Vrints CJ**, **Conraads VM**. Exercise training improves function of circulating angiogenic cells in patients with chronic heart failure. *Basic Res Cardiol* 105: 665–676, 2010.

149. **Vasa M**, **Fichtlscherer S**, **Aicher A**, **Adler K**, **Urbich C**, **Martin H**, **Zeiher AM**, **Dimmeler S**. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circulation Research* 89: E1–7, 2001.

150. **Viemann D**, **Barczyk K**, **Vogl T**, **Fischer U**, **Sunderkötter C**, **Schulze-Osthoff K**, **Roth J**. MRP8/MRP14 impairs endothelial integrity and induces a caspase-dependent and -independent cell death program. *Blood* 109: 2453–2460, 2007.

151. **Viemann D**. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. *Blood* 105: 2955–2962, 2005.

152. **Vogel RA**, **Corretti MC**, **Plotnick GD**. A comparison of brachial artery flow-mediated vasodilation using upper and lower arm arterial occlusion in subjects with and without coronary risk factors. *Clin Cardiol* 23: 571–575, 2000.

153. **Vogl T**, **Gharibyan AL**, **Morozova-Roche LA**. Pro-Inflammatory S100A8 and S100A9 Proteins: Self-Assembly into Multifunctional Native and Amyloid Complexes. *IJMS* 13: 2893–2917, 2012.

154. **Vogl T**, **Ludwig S**, **Goebeler M**, **Strey A**, **Thorey IS**, **Reichelt R**, **Foell D**, **Gerke V**, **Manitz MP**, **Nacken W**, **Werner S**, **Sorg C**, **Roth J**. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. *Blood* 104: 4260–4268, 2004.

155. **Vogl T**, **Tenbrock K**, **Ludwig S**, **Leukert N**, **Ehrhardt C**, **van Zoelen MAD**, **Nacken W**, **Foell D**, **van der Poll T**, **Sorg C**, **Roth J**. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* 13: 1042–1049, 2007.

156. **Volaklis KA**, **Tokmakidis SP**, **Halle M**. Acute and chronic effects of exercise on circulating endothelial progenitor cells in healthy and diseased patients. *Clin Res Cardiol* 102: 249–257, 2012.

157. **Voronov E**, **Carmi Y**, **Apte RN**. The role IL-1 in tumor-mediated angiogenesis. *Front Physiol* 5: 114, 2014.

158. **Vrtovec B**, **Poglajen G**, **Lezaic L**, **Sever M**, **Domanovic D**, **Cernelc P**, **Socan A**, **Schrepfer S**, **Torre-Amione G**, **Haddad F**, **Wu JC**. Effects of intracoronary CD34+ stem cell transplantation in nonischemic dilated cardiomyopathy patients: 5-year follow-up. *Circulation Research* 112: 165–173, 2013.

159. **Wang C**. Mechanical, cellular, and molecular factors interact to modulate circulating endothelial cell progenitors. *AJP: Heart and Circulatory Physiology* 286: H1985–H1993, 2004.

160. **Wang J**, **Zhang S**, **Rabinovich B**, **Bidaut L**, **Soghomonyan S**, **Alauddin MM**, **Bankson JA**, **Shpall E**, **Willerson JT**, **Gelovani JG**, **Yeh ETH**. Human CD34+ Cells in Experimental Myocardial Infarction: Long-Term Survival, Sustained Functional Improvement, and Mechanism of Action. *Circulation Research* 106: 1904–1911, 2010.

161. **Wang J-S**, **Lee M-Y**, **Lien H-Y**, **Weng T-P**. International Journal of Cardiology. *International Journal of Cardiology* 170: 315–323, 2014.

162. **Wang L**, **Luo H**, **Chen X**, **Jiang Y**, **Huang Q**. Functional Characterization of S100A8 and S100A9 in Altering Monolayer Permeability of Human Umbilical Endothelial Cells. *PLoS ONE* 9: e90472, 2014.

163. **Wang Y**, **Fang C**, **Gao H**, **Bilodeau ML**, **Zhang Z**, **Croce K**, **Liu S**, **Morooka T**, **Sakuma M**, **Nakajima K**, **Yoneda S**, **Shi C**, **Zidar D**, **Andre P**, **Stephens G**, **Silverstein RL**, **Hogg N**, **Schmaier AH**, **Simon DI**. Platelet-derived S100 family member myeloid-related protein-14 regulates thrombosis. *J Clin Invest* 124: 2160–2171, 2014.

164. **Weil BR**, **PhD EJK**, **MS KJD**, **MS JJG**, **MD BLS**, **PhD CAD**. T Cells, Endothelial Function and Cardiovascular Risk. *"Heart, Lung and Circulation"* 20: 659–662, 2011.

165. **Witkowski S**, **Jenkins NT**, **Hagberg JM**. Enhancing treatment for cardiovascular disease: exercise and circulating angiogenic cells. *Exerc Sport Sci Rev* 39: 93–101, 2011.

166. **Witkowski S**, **Lockard MM**, **Jenkins NT**, **Obisesan TO**, **Spangenburg EE**, **Hagberg JM**. Relationship between circulating progenitor cells, vascular function and oxidative stress with long-term training and short-term detraining in older men. *Clin Sci* 118: 303–311, 2009.

167. **Woodfin A**, **Voisin MB**, **Nourshargh S**. PECAM-1: A Multi-Functional Molecule in Inflammation and Vascular Biology. *Arteriosclerosis, Thrombosis, and Vascular Biology* 27: 2514–2523, 2007.

168. **Xu X**, **Ying Z**, **Cai M**, **Xu Z**, **Li Y**, **Jiang SY**, **Tzan K**, **Wang A**, **Parthasarathy S**, **He G**, **Rajagopalan S**, **Sun Q**. Exercise ameliorates high-fat diet-induced metabolic and vascular dysfunction, and increases adipocyte

progenitor cell population in brown adipose tissue. *AJP: Regulatory, Integrative and Comparative Physiology* 300: R1115–R1125, 2011.

169. **Yang H**, **Mohamed ASS**, **Zhou S-H**. Oxidized low density lipoprotein, stem cells, and atherosclerosis. *Lipids in Health and Disease* 11: 1–1, 2012.

170. **Yang Z**, **Xia W-H**, **Su C**, **Wu F**, **Zhang Y-Y**, **Xu S-Y**, **Liu X**, **Zhang X-Y**, **Ou Z-J**, **Lai G-H**, **Liao X-X**, **Jin Y-F**, **Tao J**. Regular exercise-induced increased number and activity of circulating endothelial progenitor cells attenuates age-related decline in arterial elasticity in healthy men. *International Journal of Cardiology* (September 24, 2011). doi: 10.1016/j.ijcard.2011.08.055.

171. **Yoder MC**, **Ingram DA**. Endothelial progenitor cell: ongoing controversy for defining these cells and their role in neoangiogenesis in the murine system. *Curr Opin Hematol* 16: 269–273, 2009.

172. **Yoder MC**, **Ingram DA**. The definition of EPCs and other bone marrow cells contributing to neoangiogenesis and tumor growth: Is there common ground for understanding the roles of numerous marrow-derived cells in the neoangiogenic process? *BBA - Reviews on Cancer* 1796: 50–54, 2009.

173. **Yoshioka T**, **Ageyama N**, **Shibata H**, **Yasu T**, **Misawa Y**, **Takeuchi K**, **Matsui K**, **Yamamoto K**, **Terao K**, **Shimada K**, **Ikeda U**, **Ozawa K**, **Hanazono Y**. Repair of Infarcted Myocardium Mediated by Transplanted Bone Marrow-Derived CD34 +Stem Cells in a Nonhuman Primate Model. *Stem Cells* 23: 355–364, 2005.

174. **Zhang S**, **Zhao L**, **Shen L**, **Xu D**, **Huang B**, **Wang Q**, **Lin J**, **Zou Y**, **Ge J**. Comparison of various niches for endothelial progenitor cell therapy on ischemic myocardial repair: coexistence of host collateralization and Akt-mediated angiogenesis produces a superior microenvironment. *Arteriosclerosis, Thrombosis, and Vascular Biology* 32: 910–923, 2012.

175. **Ziegelhoeffer T**. Bone Marrow-Derived Cells Do Not Incorporate Into the Adult Growing Vasculature. *Circulation Research* 94: 230–238, 2004.