SHEAR STRESS, HEMODYNAMICS, AND PROTEOLYTIC MECHANISMS UNDERLYING LARGE ARTERY REMODELING IN SICKLE CELL DISEASE

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
Wallace H. Coulter Department of Biomedical Engineering

Georgia Institute of Technology
December 2014

SHEAR STRESS, HEMODYNAMICS, AND PROTEOLYTIC MECHANISMS UNDERLYING LARGE ARTERY REMODELING IN SICKLE CELL DISEASE

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To my loving fiancée, Melissa

whose patience and dedication has always been my foundation

ACKNOWLEDGEMENTS

First, I would like to thank the members of my thesis committee for the invaluable support and feedback over the past yearsDr. Manu Platt, Dr. Betty Pace, Dr. Hanjoong Jo, Dr. Don Giddens, and Dr. Clint Joiner. Much of the success and potential impact of my thesis project would have been impossible without their individually unique, and invaluable perspectives and areas of expertise. Dr. Pace possesses a tremendous degree of passion for the field of sickle cell disease is clearly evident in her own research, and also served to inspire my project to remain focused on the clinical impact on those that suffer every day from this debilitating disease. In addition to benefitting from her knowledge and experience through our conversations and committee updates, Dr. Pace also demonstrated the very definition of collaboration and mentorship by hosting me in her lab for a week to learn from her students and fellows how to conduct the chromatin immunoprecipitation assay that later became a critical part of my thesis work. I am profoundly grateful for the opportunity to work along-side Dr. Pace and learn how that research needs be an amalgam of not only the fundamental science, but also the humanity of those that suffer from disease and disability. Dr. Paces passion and expertise were complimented by Dr. Clint Joiners piercing intellect and profound knowledge of both the clinical and scientific fields of sickle cell research. Although he joined the committee later, he wasted no time in committing his time and energy into maximizing the potential clinical impact of my research. Together, their generosity and lively discussions were of immeasurable benefit for guiding my experiments and project to ensure the highest degree of clinical translation. Dr. Giddens tremendous wealth of knowledge and understanding of both

the in vitro and in vivo biomechanical environments encouraged me to never over-assume or over-reach on my conclusions, but instead place my research in the proper context to ensure the true value could be appreciated by the field, and I will treasure the wonderful opportunity to learn from such a paramount leader of the biomedical arena. Dr. Jos voice on my committee was most valued for its capacity to encourage me to pursue more complex, interesting, and impactful mechanistic understandings of my system. In addition to his valued opinions towards shaping my thesis project, Dr. Jo also deserves special accommodation and credit for my success throughout my doctoral program as he was Manu Platts doctoral advisor, and is therefore directly responsible, in a meaningful way, in shaping the scientist and researcher I have become. Individually, each of my committee members contributed significantly to the foundation of my thesis project, and collectively the entire committee has been consistently encouraging and supportive through the completion of my project, and I thank them all for their time and commitment to my training.

Of all the people who have contributed to my growth, development, and success as a scientist and biomedical engineer, few have had a more direct and profound impact than Manu Platt. I am truly humbled by the leap-of-faith that Dr. Platt took when he first hired me as a laboratory technician in 2009, despite myself having little wet-lab training or experience. However, with a level of dedication that I have yet to see matched by any faculty member, he has tirelessly worked to ensure that I, as with all his students, achieve the highest level of scientific rigor and success. Without hierarchy, hubris, or ego, Manu has generated a laboratory culture that unites all participants, from post-doctoral fellows to high school researchers, as colleagues; he has taught us all to never dismiss another based on age of experience and encouraged every one of us to reach a potential we did not know we possessed. Our profound sense of camaraderie and family permeates our entire research space, giving credence to the concept that kindness and patience will instill a far more potent brand of loyalty

from those you work alongside, an invaluable and often too easily forgotten lesson in our ever-more competitive world. Always gracious and selfless, Manu method of leading through example has allowed me to become a more well-rounded and complete individual capable of inspiring others to work hard, able to face uncertainty and hardship head-on, and perceptive enough to understand that while one cannot succeed in a vacuum, he will thrive in a diverse and open community. Through the formative years of our lab, and as I begin my own adventure I have not, nor will never, take for granted the truly unique and inspiring experience of being the student of Manu Platt, a world-class mentor and one of my truly great friends. There are also several members of the from the BME and IBB departments, without whom, this thesis project would have not been possible. First, I would like to give a special thanks to Dr. Laura OFarrell and Kim Benjamin, who run the Physiology Research Laboratory at Georgia Tech. All the techniques I used during my animal studies I learned from the direct instruction of Dr. OFarrell, and without which it would not have been possible to as elegantly or precisely achieve the goals of my study. Dr. OFarrell was, and is, always willing to discuss new techniques, ideas, or review experimental plans to ensure the maximum benefit to every study. Despite having a tremendous amount of responsibility, Dr. OFarrells interest and passion towards animal research as a platform for advancing medicine and improving human health translates to an incredible level and quality of personal attention to any student or researcher who seeks out her expertise. Similarly, Kim Benjamin expertly runs one of the most well organized, cleanest, and highly functioning animal research laboratories I have ever seen at any institution. Kims sense of dedication and responsibility to the animal facility ensure that all animals in her facility receive the highest level of care, and are treated in the most ethical manner possible. This passion and dedication to the well-being of the animals, as well as the research projects of the laboratories, can also be found among the animal facility staff: Andrea Gibson, Altair Rivas, Ogeda Blue, Josh

Scarbrough, and Brittany Hunt. These men and women have unparalleled dedication and thoughtfulness in their duties in conducting the day-to-day care of the animals, as well as being an invaluable resource for any and all questions that researchers and students might have. I am consistently impressed by their sincere level of interest in all the different research projects, and their willingness to help out in any way they can. Furthermore, they are willing to sacrifice much of their personal lives to ensure the safety and well-being of the animals under their care: be it floods or ice storms that shut down all of Atlanta for days. Together, the entire Physiological Research Groups amazing level of collaboration, friendship, and excitement over research has made countless thesis projects, including my own, possible, and they are all invaluable members of the Georgia Tech community.

ShaAqua Asberry in the histology core of IBB was tremendously helpful in training and helping me to troubleshoot histological methods that were imperative to my research. Always with an open door, I could approach Aqua with any and all histology questions from reagents to protocols, and Aqua would do everything in her power to provide me with whatever I needed to keep the project moving forward. I am incredibly lucky to have learned histological techniques from such an expert of such a high degree. Similarly, Andrew Shaw, in the microscopy core of IBB, was instrumental in the final stages of my thesis project. Single-handedly managing four, state-of-the-art confocal microscopes, in addition to training any and all researchers is a formidable task. However, Andrew makes it all seem easy, and like Aqua, represents a fantastic resource, and a constantly welcoming and warm demeanor. Both Andrew and Aqua strive to ensure they are always available to anyone who may need their help or advice, and strive to ensure their respective cores are functioning at the highest degree possible, and their contributions to the completion of my thesis extend far beyond any simple result or scientific output.

Like any thesis project, collaboration was an integral part of the development and

execution of my research. However, I must give special thanks to everyone Sickle Cell Foundation of Georgia, who I worked collaboratively with for the duration of my doctoral thesis. While they are officially labeled as a patient advocacy group, there is no single term that can fully encompass all that they do for clinicians, researchers, and families that suffer from sickle cell disease. Fiercely passionate about those that come to them for help, the Sickle Cell Foundation of Georgia works tirelessly to help ensure the people who are affected by sickle cell disease receive all the necessary information, help, and medical care that they require. This dedication, love, and sense of duty also extend to their desire to forge a unique collaboration with our research group to help progress academic and pre-clinical studies in sickle cell disease. I would like to especially thank Irma Richard who was responsible for coordinating and recruiting all the sickle human blood donors that generously contributed to my project. Without her voice and confidence in my research project, much of what I was able to accomplish would certainly not been possible. I have never before met anyone with Irmas sense of dedication and duty to strangers who seek out her, and her colleagues, for help in such a terrifying and difficult disease. My interactions with the Sickle Foundation of Georgia have exposed me to the true humanity of this disease that is often unseen by the public, or even other researchers, and I am proud to have had the privilege to work alongside these amazing individuals who selflessly give so much to help those who suffer from sickle cell disease.

Perhaps one of the most powerful forces behind my success through my graduate career was from my beautiful fiance, Dr. Melissa Kinney. Always available with a kindness, encouragement, and a unique ability to place any problem, issue, or setback into a manageable perspective, I cannot adequately express the magnitude of my gratitude towards Melissa. There is a saying that behind every great man is a great woman, and while I do not consider myself to be a man of note, I am truly lucky to have found such a wonderful, loving, and dedicated woman to share this, and

every, adventure with. An amazingly accomplished researcher and scientist in her own right, Melissa not only was eternally available for brainstorming, and ideation, she was also astoundingly imaginative and resourceful in finding ways to reorganize and represent confusing or complicated data in the most straightforward and easy to understand ways. While this doctoral thesis is a tremendous accomplishment, my decision to attend graduate school in the beginning, and the motivation that propelled me through the program all sprung from Melissas love, compassion, and support, and without reservation or platitude, none of this would have been possible without her.

Throughout my tenure with the Platt lab, the other graduate students and post-doctoral fellows were a constant source of love, support, kindness, and entertainment. I am incredibly indebted to Catera Wilder, Ivana Parker, and Keon-young Park who helped forged the lab into the collaborative and innovative powerhouse it is today. Catera has always served as fusion of a watchful, yet powerful motherly figure; always willing to help whomever needs assistance, give advice and guidance, but also had a potent presence to ensure that everyone followed the rules. Catera was also the most diligent and talented graduate mentor that we have had in the lab, overseeing multiple undergraduate and high school researchers simultaneously, while also managing her own research project. Though for every instance where Catera was serving to maintain order in the lab, she also served to keep lab morale high through her spontaneous songs and lab dance parties, and was often aided by her accomplice Ivana. Perhaps one of the most consistently happy, cheerful, and upbeat person I have ever met, Ivana has been the unofficial morale officer of the entire lab. Whether through her free-style lyrics, insistence on happy music being played at all times, or unwaveringly supportive attitude, Ivana always succeeds in ensuring that everyone around her remains happy and positive. I have learned a great deal from Ivana on how to remain positive, hopeful, to lean on my friends and family through the various trials and tribulations of my thesis. I will endeavor to keep her positive attitude and outlook as I progress

into my career.

Along with a very communal environment in the laboratory, Keon-young Park could always be trusted to be the designated social chair of the lab. Dr. Park was always thinking of the next social event or lab outing our lab should attend, which always was certain to be a memorable time. From our yearly lab Thanksgiving and holiday parties, to sampling some of the finest restaurants in the Atlanta area, Keon-youngs invitations for good company and good food are one of the main reasons our lab functions more as a family than as a group of co-workers. In addition to her commitment to being social, Keon-young was also one of the most diligent and dedicated scientists I have ever had the pleasure to work alongside. Always asking the difficult and probing questions, while never being discouraged by complications or setbacks, Keon-young was a paragon of achievement and maintaining a healthy work-life balance that I have, and will continue, to try and emulate. I also owe an incredible debt of gratitude to Dr. Kristi Porter and her incredible contributions to the growth, development, and continued success of the Platt Lab. She has consistently served as a valuable resource for knowledge, and a reliable scientist for the conception, design, and execution of many of the protocols and projects that have become cornerstones of our lab. It was very helpful to have Kristi around to help guide me through many aspects of the thesis process, and I am extremely grateful for her tutelage. Her patience and willingness to always help younger students and take on additional mentees has taught me the responsibilities, and benefits, that become available once you have completed your thesis. Both the lab and I are extremely lucky to have Kristi as an experienced, dedicated, and nurturing participant in the lab community.

In addition to the young women how helped establish and build this lab, I have also had the distinct pleasure of watching two new classes on incoming graduate students join our ranks. Christian Rivera and Meghan Ferrall were the first of the new recruits to arrive, and I am consistently impressed with their adaptability and willingness

to meet new challenges, regardless of where they arise. For Chris, I am consistently astounded by the simultaneous number of project he is working on, without complaint or trepidation. To be a successful member of the Platt Lab, one must be comfortable with the spontaneity and creativity that Manu brings, and Chris skillfully adapts, quickly learns all the necessary skills, and produces exemplary results. His dedication to learning advanced computation fluid analysis, in addition to his synthetic chemistry will be invaluable to future students. Similarly, Meghan joined our group ready to learn whatever was necessary to get the job done. Never intimidated, she has continuously demonstrated an aptitude for both computational and traditional wet-lab protocols. Her attention to detail and organization are some of the best I have ever seen, which ensures she will have an impactful and highly successful career as a biomedical engineer. However, it is her positive and cheerful attitude, and generous soul that is a great benefit to the lab. It never ceases to amaze me her ability to find time for others, both in lab and through her numerous outreach programs. As myself, and the other senior students, begin to depart the lab, I am quite comfortable knowing that Chris and Meghan will be more than capable of continuing the mission and the spirit of that we have cultivated over the past 6 years.

Akia Parks and Andrew Shockey, while being the newest graduate students to join the lab, I have been extremely impressed with their ingenuity and willingness to tackle extremely challenging collaborative projects. Anyone who has relied on academic collaborative efforts in an aspect of their thesis project understands the unique set of challenges that can confound even seasoned researchers. However, both Andrew and Akia have demonstrated a unique resilience and endurance in spearheading two projects that heavily rely on the collaboration of other labs. In addition to their formidable wills, they have also quickly adapted and embraced the diverse culture of the Platt Lab. Both have also shown a considerable talent for mentorship and outreach by taking undergraduate and high school students at an extremely early

point in their graduate careers. I would like to thank both Akia and Andrew for preserving and elevating the sense of family and community that is found in the Platt Lab, and I am confident they will accept the mantle of leadership as they progress through their thesis with grace and fierce determination.

In addition to the outstanding graduate students I have had the pleasure to work with, I have also had the amazing opportunity to help mentor several undergraduate and high school research students. First and foremost would be my undergraduate student, Suhaas Anbazhakan, who joined our research group as a sophomore and has consistently exceeded every expectation held by either Manu or myself. Punctual, hardworking, incredibly intelligent and talented, Suhaas has an amazing capacity to observe and learn new techniques extremely rapidly. His consistency, work ethic, and attention to detail have made the final years of my thesis incredibly easy. It has been a real joy and a true honor to be Suhaas mentor, and I am confident that he will achieve remarkable things as he continues his education through Georgia Tech and beyond. I have learned an immense amount from Daniel Clough, Charlene Walton, Solomon McBride, Katrina Burch, and all the other undergraduates and high school students who have come through our lab over the years, as well as the high school researchers who are a true testament to what can be achieved regardless of circumstances. With all of their help and through our interactions, I have become a more effective motivator and mentor, and I am thankful to each of them for teaching me how to help others.

In addition to those students who I work alongside every day in the Platt Lab, there were many other scientists and researchers that greatly contributed to my academic success. I would like to thank Melissa Kemp and the entire Kemp Lab over the past five years. As our neighbors in IBB, the Kemp was always extremely welcoming and friendly to everyone in the Platt Lab from the very first day we opened our doors. I would especially like to thank Linda Kippner, an amazing woman and scientist, who was always available to answer my questions on how to properly run,

stock, and organize our lab when we were first getting started. Linda remained an invaluable resource through the years, and was always more than willing to help our lab with anything we might need, be it reagents, equipment, or protocols. This sense of an open and collaborative community was made possible also by the generosity and kindness of Dr. Maggie Gran, Ariel Kniss, Adam Prasanphanich, Dough White, and Chad Glen in the Kemp Lab. Additionally, I would like to thank Dr. Randy Ankeny and Dr. Robert Nerem for all their friendship, guidance, and help of both myself and Manu through the years. Randy was invaluable in helping with the design, construction, and troubleshooting of the shear stress system that has become a piece of equipment of paramount importance. Dr. Nerem, a legendary figure in the field of Biomedical Engineer, was, and is, absolutely remarkable in his availability to offer guidance, advice, and friendship to any who seek it; I will always be grateful for all Dr. Nerem has taught me and helped me achieve. My current accomplishments would, of course, been impossible without the unconditional patience, love, and support of my family through my entire educational career. My parents, Mike and Debbie Keegan, have always encouraged me to obtain the highest quality and degree of education possible, and have never wavered in their belief that I could accomplish amazing things. I would also like to thank my older brother, Brian Keegan, and my sister-in-law, Ricarose Roque, for their support, love, and commiseration through this entire process. Having obtained his PhD as well, Brian was always a welcome shoulder to vent the shared frustrations of the academic process and helped bring much needed stability to these past few years.

Finally, I would like to thank the funding sources that provided the monetary support to make this research possible. A special thanks to the National Science Foundation Graduate Research Fellowship for providing funding for me, and many other graduate students, to conduct cutting-edge and translational research. Additionally, I would like to thank the Whitaker International Fellowship Program for

providing the means to conduct a 2-month research project in Johannesburg, South Africa; through the program, I met some amazing scientists, friends, and learned firsthand some of the incredible challenges that face the global health field. Lastly, I would like to thank the National Science Foundation for the Young Investigators Award that provided the bulk funding for my entire research project.

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LIST OF SYMBOLS OR ABBREVIATIONS

AA	normal hemoglobin.
Akt	protein kinase B.
AP-1	activator protein 1.
ChIP	chromatin immunoprecipitation.
DAPI	4',6-diamidino-2-phenylindole.
DNA	deoxyribonucleic acid.
EC	endothelial cell.
ECGS	endothelial cell growth supplement.
EGTA	ethyleneglycoltetraacetic acid.
ELISA	enzyme linked immunosorbent assay.
ERK	extracellular-signal regulated kinase-1/2.
FBS	fetal bovine serum.
HAEC	human aortic endothelial cell.
ICAM-1	intercellular adhesion molecule-1.
JNK	c-jun N-terminal kinase.
MCA	middle cerebral artery.
MRI	magnetic resonance imaging.
mRNA	messenger ribonucleic acid.
μCT	micro computed tomography.
NBF	neutral buffered formalin.
NFκB	nuclear factor-κB.
NO	nitric oxide.
PBMC	peripheral blood mononuclear cell.
PBS	phosphate buffered saline.
PFA	paraformaldehyde.

PMSF	phenylmethane- sulfonyl phenylmethylsulfonyl fluoride.
RT-PCR	reverse transcriptase polymerase chain reaction.
SCD	sickle cell disease.
SDS	sodium dodecyl sulfate.
SEM	standard error of the mean.
SMC	smooth muscle cell.
SS	sickle hemoglobin.
TCD	transcranial doppler.
TNFα	tumor necrosis factor α.
VCAM-1	vascular cell adhesion molecule – 1.

SUMMARY

Clinically described for over a century, sickle cell disease is a genetic disorder affecting 100,000 people in the US and millions worldwide. While the mutation only affects a single protein (hemoglobin) that is expressed in a single cell type (red blood cells), sickle cell disease has devastating effects throughout the body, significantly reducing the lifespan of those afflicted. One significant comorbid condition is the 200-fold increase in stroke risk in children under the age of 20, with the highest risk between 2 and 5 years of age. Although clinicians have demonstrated that the increased stroke risk is attributed to severe remodeling of the cerebral arteries, the underlying cause of this remodeling remains unknown. In fact, the only treatment option available for children with sickle cell disease is monthly blood transfusions, which carries life-threatening risks for alloimmunity and iron overload. Therefore, there is a clear need to develop a more complete, mechanistic understanding of stroke in sickle cell disease. To address this need, my research investigates the unique circulatory environment of sickle cell disease and mechanisms promoting rapid arterial remodeling leading to stroke.

Clinical descriptions of the remodeled cerebral arteries in patients with sickle cell disease reveal elastin degradation, neointimal formation, and luminal narrowing. Such arterial remodeling exhibits similarities to other cardiovascular diseases, including atherosclerosis, which are characterized by changes in vessel structure, concomitant with protease-mediated remodeling of the extracellular matrix (Platt et al. Am J Physiol Heart Circ Physiol. 2007). Of particular interest are the cathepsins K and V, powerful enzymes that degrade collagen and elastin. In order to study the proteolytic

response in sickle cell disease, our lab developed the multiplex cathepsin zymography technology, which quantifies of the activity of multiple enzymes with high specificity and sensitivity (Li et al. Anal Biochem 2010 & Wilder et al. Arch Biochem Biophys 2011).

We have demonstrated that the inflammatory cytokine TNFα is sufficient to increase the activity of cathepsins K and V in endothelial cells (Keegan et al. Mol Cell Biochem. 2012). This is particularly relevant because sickle cell disease presents as a chronic inflammatory syndrome, with a 20-fold increase in serum TNFα (Keegan et al. Anemia. 2012). Therefore, the vasculature in sickle cell disease may be preconditioned by the circulatory milieu to induce cathepsin-mediated remodeling. The predisposition for remodeling is also aggravated by circulating monocytes in sickle cell disease; these monocytes exhibit the unique ability to induce cathepsin K activity in endothelial cells, independent of TNFα (Keegan et al. Anemia. 2012). Together, these findings indicate that the circulatory environment of sickle cell disease contains powerful, pro-cathepsin activation factors, which may be responsible for the degree and speed of remodeling in the cerebral vasculature.

Cathepsins K and V are also sensitive to changes in vascular hemodynamics. In healthy individuals, pro-remodeling shear stress is generally restricted to regions where arteries bifurcate; however, in sickle cell disease, aggregations of red and white blood cells can spontaneously form throughout the vascular tree and impede blood flow, leading to spontaneous, localized regions of pro-remodeling shear stress. Through the implementation of a custom shear stress bioreactor, we determined that endothelial cells exposed to oscillatory, pro-remodeling shear stress significantly increase cathepsin K activity, independent of TNFα activity (Keegan et al. in prep). Conversely, unidirectional, vasoprotective shear stress reduced cathepsin K activity below detectable levels, even after stimulation with TNFα. Therefore, the propensity

for spontaneous perturbation of vascular hemodynamics in sickle cell disease exacerbates the already profound risk for cathepsin-mediated remodeling.

To more mechanistically uncover the biochemical pathways regulating the cathepsin response to shear stress, monocyte adhesion and TNFα stimulation, we examined the phosphorylation state of several intracellular kinases. Of several kinases assayed, the phosphorylated isoforms of JNK and its downstream target c-Jun were significantly increased, suggesting a role for the MAPK pathway in mediating cathepsin activity under inflammatory stimuli. The MAPK pathway was further implicated when inhibition of JNK signaling significantly reduced (45-65%) cathepsin K and V activity after TNFα or sickle mononuclear cell stimulation (Keegan et al. Anemia 2012). Interestingly, despite their effects on cathepsin activity, neither vasoprotective nor pro-remodeling shear stress induced changes in the phosphorylation of JNK or c-Jun, suggesting divergent biochemical and biomechanical pathways regulating remodeling responses in sickle cell disease (Keegan et al. in prep).

Our studies have identified constituents of the MAPK/JNK pathway as druggable targets for the prevention of stroke in sickle cell disease. Therefore, we have employed a transgenic mouse model that expresses human sickle hemoglobin, thus recapitulating the multi-faceted pathology of sickle cell disease, including the first identification of stroke lesions (Keegan et al. in prep). Primary cerebral cells from sickle transgenic mice also exhibited higher levels of cathepsin activity, compared to wild type controls. The cerebral and carotid arteries of sickle transgenic animals displayed evidence of significant elastin fragmentation and remodeling which co-localized with increased cathespin K expression within the arterial wall; *in vivo* inhibition of JNK substantially reduced this expression as well as decreased the number of elastin breaks in the elastic lamina. Ongoing work aims to conduct additional drug trials to specifically inhibit active cathepsin, with the hypothesis that specific targeting of cathespin K activity will prevent the vasculopathy associated with sickle cell diseas thereby reducing the

risk of stroke.

The etiology of stroke in sickle cell disease is complex, resulting from a "perfect storm" of individual biological, biochemical, and biomechanical factors capable of uniquely and synergistically promoting cathepsin activity. Together, this project has established a robust, mechanistic, and multi-scale approach for studying remodeling in response to physical and chemical stimuli, *in vitro* and *in vivo*, which will ultimately enable the development of novel, targeted therapeutics for sickle cell disease, as well as countless other clinically described systemic remodeling pathologies.

CHAPTER I

INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder that causes polymerization of hemoglobin molecules within red blood cells, deforming them into the canonically described "sickle" shape, resulting in a 220-fold increase in the risk of stroke for children under the age of 16. Seminal studies have concluded that increased blood velocities in the middle cerebral artery (MCA) of children with sickle cell disease strongly correlated to an increased risk for stroke lesion formation. These lesions show advanced arterial remodeling, characterized by changes in extracellular matrix composition, cell organization, and cell phenotypes that induce chronic effects on the structure, mechanical properties, and overall vessel health; however, the underlying mechanisms governing this process remain unknown. Traditionally, studies of sickle cell cardiovascular pathologies are limited to low shear, low oxygen, venous-side capillaries. However, stroke in sickle cell disease originates in large, well-oxygenated arteries exposed to high shear stress. Endothelial cells normally maintain arterial homeostasis, but in pathological states they respond to monocyte adhesion, inflammatory cytokines, and low and oscillatory fluid shear stress. In response, endothelial cells promote maladaptive remodeling of the artery, leading to luminal narrowing and restricted blood flow. One way this is accomplished is by increasing secretion of cathepsins, powerful cysteine proteases, and downregulation of protease inhibitors. Two cathepsins, cathepsins K and V, are increased during arterial remodeling, and represent the most powerful elastase and collagenase, respectively, yet identified. In sickle cell disease, pro-remodeling stimuli, including chronically elevated plasma levels of tumor necrosis factor alpha (TNFα), cause systemic activation of the endothelium and induce

monocyte adhesion. Simultaneously, aggregations of rigid, sickled red blood cells spontaneously form along the endothelium, disturbing flow and inducing low or oscillatory fluid shear stress. Alterations in hemodynamic shear stress due to these aggregations potentially occur throughout the vascular tree, rather than being limited to regions near branches or bifurcations, as seen in individuals without sickle cell disease.

The objective of this proposal is to develop a mechanistic understanding of how the sickle mutation induces a cardiovascular syndrome that promotes arterial remodeling. The central hypothesis is that disturbed flow, chronic inflammation, and elevated monocyte adhesion due to sickle cell disease increase cathepsin-mediated arterial remodeling contributing to increased risk for stroke. The central hypothesis will be tested through following aims (Fig 1-1):

Specific Aim 1: Determine the combinatory effects of TNFα stimulation and monocyte adhesion on large artery endothelial cell (EC) cathepsin activity. Hypothesis: Monocyte adhesion and TNFα stimulation will induce cathepsin activity in large artery endothelial cells. People with sickle cell disease have increased numbers of circulating monocytes and chronically elevated plasma levels of TNFα. We will investigate how these inflammatory mediators independently and co-operatively increase cathepsin activity in large artery endothelial cells. EC cultures will be maintained with or without TNFα, as well as co-cultured with primary monocytes isolated from whole blood of individuals with either normal (AA) or sickle (SS) β-globin. This study will allow us to parse out critical stimulators of cathepsin activity, as well as the intracellular signaling cascade that transduce extracellular, inflammatory signals circulating in the blood milieu of sickle cell disease into increased cathepsin production.

Specific Aim 2: Examine the extent to which low or oscillatory shear stress observed in sickle cell disease exacerbates cathepsin activity induced by inflammatory factors and monocyte-endothelial cell interactions. Hypothesis: Low or oscillatory shear stress characteristic of arterial stenosis in sickle cell disease will promote greater amount of monocyte adhesion and cathepsin activity compared to unidirectional shear stress. In sickle cell disease, disruption of cerebral blood flow profiles may accelerat arterial remodeling by upregulating cathepsin activity, which have been shown to be shear regulated, leading to stroke lesion formation. Large artery endothelial cells will be stimulated with or without TNFα, as well as co-cultured with primary monocytes isolated from AA or SS individuals and placed in a cone-and-plate shear system, which will impart either pulsatile, unidirectional shear stress characteristic of a normal cardiac cycle, or low or oscillatory shear stress characteristic of arterial narrowing in sickle cell disease. This study aims to elucidate how the superposition of low or oscillatory shear stress on the underlying biochemical and cell-cell interactions in sickle cell disease increase cathepsin activity in large artery endothelial cells.

Specific Aim 3: Investigate the role of the JNK/c-jun signaling axis in cathepsin-mediated arterial remodeling and stroke lesion formation in sickle transgenic mice. Hypothesis: Inhibition of JNK and c-jun singling in sickle transgenic mice will reduce in vivo cathepsin K and V activities, thereby preserving structural integrity of cerebral and carotid arteries and reducing the incidence of stroke. The sickle transgenic mice used in this study have been genetically modified to exclusively express human hemoglobin, specifically normal or sickle human β-globin, thereby effectively recapitulating the multifactorial sequelae observed in human patients. Transgenic mice will be treated with or without an inhibitor of JNK activity for 8 weeks. Animal brains will be isolated and imaged using a 9T animal

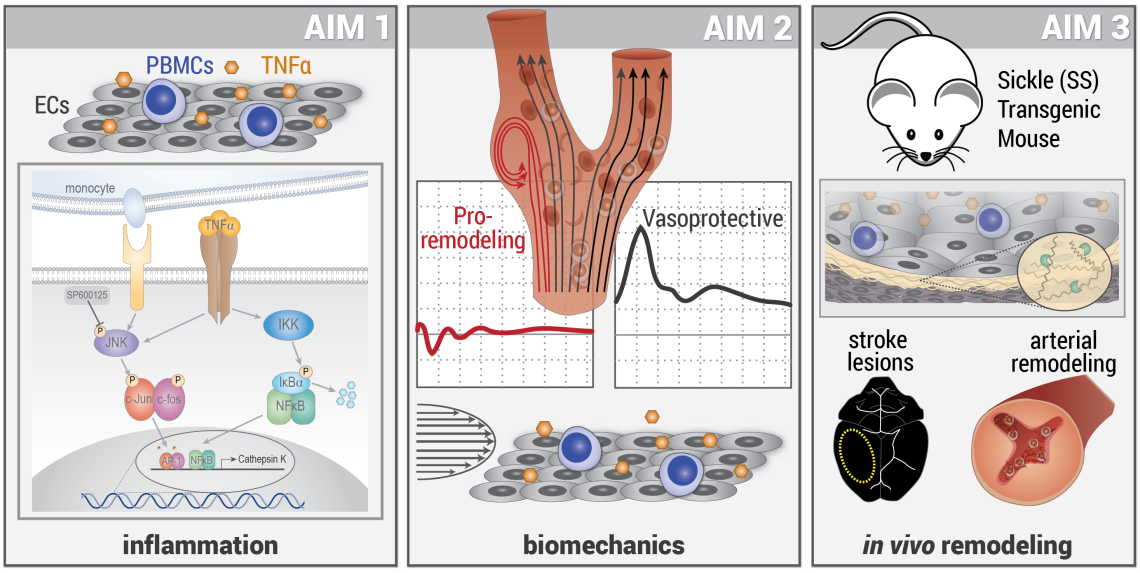


Figure 1-1: Research aims schematic overview

MRI system to identify and quantify sites of stroke lesions. Subsequently, cathepsin activity and fragmentation of the elastic lamina of the internal carotid and middle cerebral arteries will be quantified from these animals, compared to vehicle treated controls. This study aims to specifically determine the influence of the JNK/c-jun axis on cathepsin-mediated arterial remodeling leading to stroke in sickle cell disease.

This proposal is innovative because it seeks to elucidate a currently unknown fundamental, mechanistic understanding of the initiation and progression of large artery remodeling in sickle cell disease. Currently, there are limited treatment options available to detect, prevent, or mitigate the development of lethal cardiovascular pathologies. However, this proposed work aims to analyze the potential of biochemical and biomechanical factors characteristic to sickle cell disease to induce remodeling of large arteries, and identify novel, therapeutic targets to improve the quality of life of people living with this life-shortening disease.

CHAPTER II

BACKGROUND

2.1 Significance

Sickle cell disease affects nearly 100,000 people in the United States, and millions more across the globe. Of the multitude of pathologies and syndromes associated with sickle cell disease, perhaps one of the more devastating is the development of stroke in young children. By the age of 20, 11% of children suffering from sickle cell disease will suffer a major, clinically aparent stroke [97]. A further 17% to 35% of affected children will suffer an asymptomatic, silent, infarcts that can result in cognitive defects and learning disabilities later in life [94, 107, 125, 144]. Additionally, the risk of first stroke is highest during the first decade of life, specifically between the ages of 2 and 5 years [97, 133, 35]. Pioneering work done by the Stroke Prevention Trial in Sickle Cell Anemia (STOP) has shown that elevated middle cerebral blood flow velocities greater than 200 cm/sec are highly predictive of stroke risk, which can be mitigated with monthly blood transfusions [2, 4]. Unfortunately, regular monitoring of cerebral blood flow remains difficult at the clinical level, and regular blood transfusions carry significant risks of infection, alloimmunity, and iron overload [143, 133, 4]. Additionally, hydroxyurea remains the only approved drug specifically approved by the FDA for the management and mitigation of symptoms associated with sickle cell disease, yet has been shown to be ineffective with reduction of risk of stroke [39, 130, 145]. Therefore, there is a pressing need for improved for long-term screening and/or treatment of stroke in sickle cell disease.

One of the complicating factors in developing novel therapeutics and diagnostic technologies is the lack of a mechanistic understanding of stroke lesion formation or

progression. Magnetic resonence angiograms of stroke victims with sickle cell disease showed substantial reduction of blood perfusion through the carotid and middle cerebral arteries. Furthermore, the vascular occlusions were shown to be the result of luminal narrowing due to advanced arterial remodeling, characterized by neoimtimal formation due to proliferation of endothelial and smooth muscle cells, monocyte infiltration, excess matrix deposition, and elastic lamina degradation. The remaining luminal area becomes further occluded due to aggregations of circulating mononuclear cells, red blood cells, and activated platelets [153, 133, 113]. While there are several working hypotheses describing the luminal aggregation of circulating blood cells, arterial remodeling in the context of sickle cell disease remains understudied. The research presented here is significant because it aims to develop a mechanistic understanding of how the inflammatory circulatory environment unique to sickle cell disease promotes rapid remodeling of large arteries, in order to identify new therapeutic targets for the prevention of stroke lesion formation.

2.2 Sickle Cell Disease

2.2.1 Molecular Basis

Sickle cell disease is a genetic condition that is caused by substitution of valine for glutamic acid in the sixth position of β-globin, a constituent protein of hemoglobin protein [57]. While glutamic acid is a polar, hydrophilic molecule, valine is nonpolar and strongly hydrophobic. When saturated with oxygen, the valine residue is shielded from the aqueous cytoplasm of the red blood cells allowing the hemoglobin molecules to remain soluble. However, deoxygenation caused by gas exchange within the microcirculation results in conformational changes in the sickle hemoglobin (HbS) molecules which expose the hydrophobic residues to the aqueous cytoplasm [11]. Hydrophobic interactions between the HbS molecules, governed by overall hemoglobin concentration, result in the formation of rigid fibers within the red blood cell [54, 24].

Through heterogeneous nucleation, valine residues along the surface of these fibers act as focal points for further nucleation and growth of adjacent fibers [38]. As these fibers grow, they distort the red blood cell membrane resulting in the canonically described "sickle" shape. As the red blood cell becomes re-oxygenated, the polymerization reaction is reversed; the hemoglobin fibers "melt" as oxygen is taken up by the HbS and the normal discoid shape returns. However, the time required to melt sickled hemoglobin polymers is greater than the time an average red blood cell spends in oxygenated circulation, which allows for a significant population of sickled red blood cells to exist in the arterial circulation [95].

One of the more well-characterized presentation of sickle cell disease is the vaso-occlusive, or pain, crisis. Normally, the bi-concave discoid shape, excess plasma membrane, and lack of a rigid nucleus allows red blood cells to be highly deformable thereby permitting them to pass through the microcirculation [123]. However, the formation of the HbS fibers in sickle red blood cells during gas exchange negates this capacity for extreme deformation. As a result, sickled red blood cells can become trapped in the microcirculation restricting or blocking blood flow. The etiology of sickle cell vaso-occlusion begins with the heterogeneity of the red blood cell population. Sickle red blood cells exist in four distinct fractions, with the more immature cells, reticulocytes, being the most adherent [64, 62]. As sickled reticulocytes begin accumulating along the endothelium, larger, less deformable cells, such as circulating monocytes, become entrapped. The resulting cell aggregation further restricts blood flow until complete occlusion is achieved [65, 40]. Although classically described in the microcirculation, pathological RBC adhesion to vascular endothelial cells can potentially occur throughout vascular system. One of the significant causes of these vascular occlusions originates with the pathological interactions between circulating RBCs and other vascular cells.

2.2.2 Chronic Inflammation as a Co-Morbidity of Sickle Cell Disease

Although sickle cell disease is canonically classified as a genetic hematological disorder, the associated complications that arise from pathological interactions between RBCs, vascular endothelial cells, and circulating mononuclear cells results in a chronic inflammatory state, evidenced by the abnormal presence of circulating endothelial cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin in the plasma [14], as well as elevated plasma levels of inflammatory cytokines like TNFα and IL-1 [104, 86]. Direct interactions between RBCs and circulating monocytes results in monocytes acquiring an activated phenotype, as shown by the expression of activated integrins [82], and cytokine-inducible CD64 [36], by increased release of leukocyte elastase, and by increased shedding of L-selectin and CD16 [75, 14, 153]. The perpetual activated phenotype of the monocytes, increase in circulating monocyte numbers, and elevated circulating inflammatory cytokines results in a chronically activated endothelium that promotes pathological monocyte-endothelial cell interactions. Such interactions are also independently exacerbated via direct interactions between endothelial cells and sickle RBCs. Cyclic sickling of the RBC membrane causes membrane inversion, leading to pathological adhesion to endothelial cells [11], especially among the immature reticulocytes, which damages the endothelium [14] causing elevated EC expression of VCAM-1, ICAM-1, P-selectin, and E-selectin [46, 64, 22]. Ultimately, the circulatory milieu of people with sickle cell disease maintains a chronic activation of endothelium throughout the vascular tree and promotes monocyte-EC interactions which represents an initial stage in many pathological vascular remodeling pathologies.

2.3 Stroke in Sickle Cell Disease

2.3.1 Prevalence of stroke in SCD

While the vaso-occlusive crises of the microvasculature represent a significant co-morbidity associated with sickle cell disease, aggregations of red blood cells, alone, are insufficient to explain the clinical reality that children with sickle cell disease have a 221-fold increase in stroke risk and a 410-fold increase in cerebral infarction specifically; the risk of first stroke is highest during the first decade of life, specifically between the ages of 2 and 5 years [97, 133, 35]. In addition to the devastating strokes with clinically detectable symptoms, 17% to 35% children with sickle cell disease also suffer from numerous silent, or unsystematic strokes [94, 107, 125], resulting in cognitive defects and learning disabilities that are undetected until later in the child's development [144]. Additionally, children that have had previous silent infarcts are at higher risk of future strokes in terms of both covert and silent cerebrovascular events [94, 92]. The risk factors for silent infarction are generally different from those for clinical stroke with the only identified laboratory predictors being raised white blood-cell count and the SEN βglobin haplotype [69].

2.3.2 Characteristics of stroke lesion formation

Autopsies and angiographs in the 1970s and 1980s of patients with SCD who died or suffered clinically presenting strokes showed narrowing, and sometimes complete occlusion, of the large cerebral arteries that was associated with intimal hyperplasia [19, 53, 47, 120, 127, 53]. The regions of luminal narrowing often had thickening caused by fibrous connective tissue and smooth muscle cell proliferation with reduplication and fraying of the internal elastic lamina [138]. As mentioned previously, people with sickle cell disease are also at elevated risk of silent strokes, which are likely caused by ischemic changes of the frontal lobes, which do not cause motor or sensory deficits. However, the majority of patients with silent stroke have normal

large vessel histology and angiography, yet had multiple sites of small vessel disease resulting in focal areas of necrosis and/or hemorrhage [107, 19]. While the histological examinations of stroke formation have been previously investigated, there is a pressing need to more completely underestand the cellular and systemic mechanisms that initiate and regulate progression of vascular lesions leading to stroke in sickle cell disease. However, recent studies have identified a possible, positive feedback loop, that may predispose the large cerebral vessels to stroke lesion formation. The endothelium of the cerebral vasculature exists in a perpetually activated state in response to elevated circulating inflammatory cytokines, which, in turn, promotes adhesion of circulating monocytes and RBCs [133, 53, 153]. Simultaneously, cyclic sickling of erythrocytes causes hemolysis, in which damaged red blood cells release hemoglobin into the blood, and its reactive heme group scavenges nitric oxide (NO), inhibiting flow-mediated vasodilation and exacerbating the luminal narrowing [133]. The byproducts of hemolysis and inflammation act to positively feedback increasing systemic levels of inflammatory cytokines, monocyte mobilization, and pathological monocyte-endothelial [30, 133, 10]. However, the chronic inflammation, elevated monocyte-endothelial interactions, and elastin fragmentation clinically described in people with sickle cell disease are similar to what has been observed during other cardiovascular remodeling pathologies, such as atherosclerotic plaque development. Therefore, it is plausible that that common mechanisms for arterial remodeling may exist between the well-studied, well-characterized atherosclerosis and the understudied mechanisms of strokes in children with sickle cell disease.

2.3.3 Treatment options and limitations

While it is difficult for doctors to provide early detection of stroke in sickle cell disease, seminal work by Adams et al. as part of the Stroke Prevention Trial in Sickle Cell Anemia (STOP) established the gold-standard for clinical evaluation of

stroke risk in sickle cell disease by statistically linking cerebral blood flow velocities greater than 200 cm/s to high stroke risk, as well as establishing monthly blood transfusions as an effective prophylactic against lesion formation[4]. Transfusions also result in reductions in middle cerebral artery velocities correlated with the pre-transfusion velocity [142]. In fact, that the progression of large-vessel stenoses can be curtailed by transfusion therapy has been corroborated through angiography [120, 133]. It is predicted that children who receive monthly blood transfusions have a 95% of remaining stroke free [106, 4]. However, monthly blood transfusions carry a significant risk of alloimmunity and iron overload, which preclude this treatment from being widely deployed across the general population of pediatric sickle cell patients [143, 133, 4].

2.4 Cathepsins and Arterial Remodeling

2.4.1 Cathepsin overview

Cathepsins, generally known as lysosomal cysteine proteases, are normally described to degrade intracellular or endocytosed proteins [141, 139, 140]. However, these proteins have recently been shown to be induced and secreted into the extracellular spaces, where they participate in pathological tissue remodeling in inflammatory and autoimmune diseases such as atherosclerosis [121, 129, 81, 58, 99, 131], obesity [149, 148, 134], rheumatoid arthritis [55, 8], cardiac repair [131], cardiomyopathy [128, 108, 124], and cancer [93, 29]. Cathepsins are synthesized as precursors that are enzymatically inactive; removal of the N-terminal propeptide by other proteinases or autocatalysis allows for exposure of the cathepsin active site allowing for substrate catalysis [72]. Cathepsins have broad substrate specificity for cell matrix components, and through the combination of different species, they can degrade nearly all intra and extracellular proteins. While highly active and extremely potent, cathepsin activity is tightly regulated extracellularly by cystatin C, which is found in all extracellular

fluids [34].

2.4.2 Cathepsins in arterial remodeling

For the purposes of this thesis, arterial remodeling will be defined as changes in the composition of proteins, cell types, and even cell phenotypes that induce chronic effects on the structure, mechanical properties, and total health of a vessel [98, 81]. This includes degradation of old matrix by newly activated proteases as well as synthesis and deposition of new extracellular matrix proteins. Cathepsins have been shown to be highly active at extracellular matrix degradation, in a variety of tissue remodeling pathologies. As discussed previously, arterial remodeling in atherosclerotic plaques shows strong parallels in structure and pathology to stroke lesions observed in children with sickle cell disease, although there are key differences. Atherosclerotic plaques form around excess lipid deposition below the endothelial layer, leading to luminal narrowing. In sickle cell disease, the "plaques" are not lipid-laden, and are thought to develop due to excess extracellular matrix deposition. Cathepsins K and V, the most powerful mammalian collagenase and elastase, respectively, have recently gained interest as mediators of vascular remodeling. Both cathepsins K and V have been identified in atherosclerotic plaques [7, 76] and in neointima following balloon angioplasty [87, 111]. Furthermore, the pathophysiological importance of cathepsin K in atherosclerosis has been demonstrated in double-knockout mice deficient in both apolipoprotein E and cathepsin K. In this model, the number and size of atherosclerotic lesions were reduced, and there were fewer breaks in the elastic lamina [129]. Several studies have shown expression of cathepsin K by vascular smooth muscle cells (SMCs) and macrophages, and their roles in vascular remodeling [147, 76, 4, 111]. Studies with human atherosclerosis samples showed a positive correlation between the cathepsin K levels in endothelium and atherosclerotic lesion development, providing supporting evidence for cathepsin K in elastic degradation [111].

2.4.3 Shear stress regulation of cathepsin activity

It has been well established that high, unidirectional fluid shear stress is critical for maintaining vascular health. Arteries exposed to low or oscillatory (OS) blood flow are more likely to develop atherosclerotic lesions, while vasculature exposed to unidirectional (USS) flow are more atheroprotected. Part of this shear-dependent plaque development may, in a significant part, be attributed to biomechanical regulation of cathepsin activity. Previous studies have shown that OS increases endothelial cell cathepsin K mRNA and protein levels and activity compared with USS, suggesting that cathepsin K is regulated by fluid shear stress [111]. Conversely, USS inhibits gelatinase and elastase activity in endothelial cells in a cathepsin-dependent manner. It has also been shown that mouse cathepsin L, the ortholog of human cathepsin V, partially contributes to the shear-dependent regulation of the extracellular matrix protease activity; knockdown of mouse cathepsin L showed a partial inhibitory effect [111, 110].

CHAPTER III

TUMOR NECROSIS FACTOR ALPHA STIMULATES CATHEPSIN K AND V ACTIVITY VIA JUXTACRINE MONOCYTE-ENDOTHELIAL CELL SIGNALING AND JNK ACTIVATION[1]

3.1 Introduction

Children with sickle cell disease have an 11% chance of suffering a major stroke by the age of 16, and pulmonary hypertension represents 20-30% of mortality due to sickle cell disease in adult patients [85, 143]. Both of these pathologies progress with severe vascular remodeling, defined as changes in the composition of proteins, cell types, and even cell phenotypes that induce chronic effects on the structure, mechanical properties, and total health of a vessel [13, 79, 88]. Histological studies of vascular remodeling in people with sickle cell disease have implicated increased monocyte infiltration into the subendothelial space, degradation of the elastic lamina, and luminal narrowing as contributors to lesion development in the cerebral vasculature that contributes to sickle strokes [113].

Sickle cell disease is a multi-factorial, genetic disorder that causes *in vivo* polymerization of hemoglobin molecules into rigid fibers within red blood cells, deforming them in the canonically described "sickle" shape. This hemoglobin polymerization is thought to occur under low oxygen, or hypoxic, environments of the postcapillary venules as a consequence of the vaso-occlusion and ischemia [11, 61, 65]. The

[1]Modified from: Keegan, P. M., Wilder, C. L., & Platt, M. O. (2012). Tumor necrosis factor alpha stimulates cathepsin K and V activity via juxtacrine monocyte-endothelial cell signaling and JNK activation. Molecular and Cellular Biochemistry, 367(1-2), 6572. doi:10.1007/s11010-012-1320-0

formation of the hemoglobin fibers, increases membrane tension and cell stiffness, significantly reducing the ability of the red blood cells to traverse the microcirculation [11]. On average, sickle red blood cells only survive 10 days, compared to the 120 days of normal red blood cells before lysing. During hemolysis, damaged red blood cells release hemoglobin into the blood, and its reactive heme group scavenges nitric oxide, inhibiting flow-mediated vasodilation and exacerbating luminal narrowing [133]. Stiff, sickled red blood cells and the byproducts of their hemolysis cause damage and increase systemic levels of inflammatory cytokines, mobilized monocytes [30], and pathological levels of increased monocyte adhesion to the endothelium [133, 10]. Furthermore, sickled red blood cells can spontaneously form aggregations in both the venus and arterial circulation, obstructing blood flow and leading to localized areas of hypoxia [10]. Taken together, the inflammation, monocyte adhesion, and vascular remodeling seen in sickle cell disease are all steps similar to atherosclerotic plaque development, suggesting that common mechanisms for arterial remodeling may exist between the well-studied, well-characterized atherosclerosis and the understudied mechanisms of strokes in children with sickle cell disease.

In atherosclerosis and other cardiovascular disease, endothelial cells initiate vascular responses to inflammatory cytokines, such as tumor necrosis factor alpha (TNFα). Activation of the endothelium results in increased surface expression of cell adhesion molecules and secretion of powerful chemokines essential for the recruitment of circulating monocytes to the vascular wall [91]. Once adhered, paracrine and juxtacrine signaling between monocytes and endothelial cells arrest monocytes along the endothelium, and permit transmigration to the subendothelial space [14, 132]. Ultimately, these inflammatory signals can initiate cellular programs to promote arterial remodeling as atherosclerotic plaques progress.

Collagen and elastin are two important extracellular matrix proteins degraded by cathepsins during atherosclerotic lesion formation [77, 83, 111]. Cathepsins are

a family of cysteine proteases that have been highly implicated in cardiovascular disease [80, 83], and endothelial cells contribute to their production and this pathology [110, 111]. Cathepsins K and V, in particular, have gained attention due to their potent proteolytic activity. Cathepsin K is both the most potent human collagenase identified, as well as an extremely powerful elastase [59], and has been shown to be highly expressed in atherosclerotic lesions [129, 111]. Cathepsin V is the most powerful mammalian elastase yet identified, and is expressed in human monocyte-derived macrophages [150]. Studies have shown that the human cathepsin V ortholog, murine cathepsin L [21, 137], significantly contributes to cardiovascular disease in mouse models [149, 70]. However, elucidating the contributions of specific cell types and their stimulation of each other is still an important goal to treat this disease.

In this study, the individual and combinatorial effects of TNFα, heme, and monocyte adhesion, and oxygen level on cathepsin activity by human aortic endothelial cells was investigated to understand how the unique circulatory environment of sickle cell disease may induce proteolytic remodeling of the arterial wall leading to stroke. This was accomplished using multiplex cathepsin zymography, a novel enzymatic activity assay developed in our lab to simultaneously quantify cathepsins K, L, S, and V expression levels of active enzyme [78, 147]. Furthermore, we investigated phosphorylation of Akt, extracellular signal-regulated kinase 1 and 2 (ERK, c-Jun N-terminal kinases (JNK), and c-jun to identify intracellular signaling cascades linking TNFα stimulation and monocyte binding to increased levels of cathepsins K and V activity to suggest a mechanism for pharmaceutical targeting.

3.2 Methods

3.2.1 Cell Culture

Human aortic endothelial cells (HAECs) (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% endothelial cell growth serum (ECGS). Human THP-1 acute monocytic leukemia cells (American Type Culture Collection [ATCC]) were cultured in RPMI medium 1640 (Mediatech) containing 10% FBS, 0.05% β-mercaptoethanol, 1% L-glutamine, and 1% penicillin/streptomycin. HAECs were transfected with cathepsin K overexpression plasmids on the pCMVSport6 background at 50-80% confluence with Lipofectin (Invitrogen) in OptiMEM according to manufacturer's instructions.

3.2.2 Monocyte adhesion and endothelial cell co-cultures

HAECs were preconditioned in the presence or absence of 10 ng/mL recombinant human TNFα (Invitrogen), 2.5μM heme (Sigma), or both for 4 hours in either normoxic (20% O_2) or hypoxic (1% O_2) conditions prior to adding 500,000 monocytes/ml. THP-1 monocytes were allowed to adhere for 45 minutes prior to washing with PBS, and co-cultures adhered to HAECs were maintained for an additional 20 hours under normoxic or hypoxic oxygen conditions. Indirect co-cultures were generated by suspending 500,000 monocytes/mL above pre-stimulated HAECs using a transwell insert with a 0.2m pore size for 20 hours. For JNK inhibition studies, HAECs were preconditioned with 10 μg/mL of SP6000125 (EMD Biosciences) for one hour prior to addition of THP-1s and co-culture.

3.2.3 Monocyte cell counts

Prior to co-culture, HAECS were stained with 5μM cell tracker red (Life Technologies) for 15 minutes followed by 3, 5 minute washes with sterile PBS. Simultaneously,

THP-1 monocytes were incubated in a 5μM dilution of cell tracker blue (Life Technologies) for 15 minutes, followed by 3, 5 minute washes. THP-1 monocytes were then allowed to adhere as described previously, and fluorescent images were taken, and cell counts were detected using automated segmentation algorithms based upon Otsu global thresholding within Cell Profiler (Broad Institute). For enhanced contrast, fluorescent images were pseudocolored such that HAECs appear blue and THP-1 monocytes appear green.

3.2.4 Multiplex cathepsin zymography

Cathepsin zymography was performed as described previously [147]. Determination of cathepsin V band required incubation in acetate buffer, pH 4. Gels were imaged using an ImageQuant 4010 system (GE Healthcare). Images were inverted in Adobe Photoshop and densitometry was performed using Scion Image.

3.2.5 *in situ* zymography

Co-cultures of HAECs and THP-1s monocytes were prepared as above; after the 20 hour incubation time, cultures were rinsed with PBS and incubated in zymography assay buffer (0.1M sodium phosphate buffer, 1mM EDTA, 2mM DTT, pH 6.0) containing 0.5mM Z-GPR-MβNA (Enzo) and 1mM 5-nitrosalicylic acid (Sigma). To isolate cathepsin K signal, serine proteases were inhibited with 1mM PMSF (Sigma), matrix metalloproteinases (MMPs) were inhibited with 10 mM EDTA (Sigma), and cathepsin B was inhibited with CA-074 (EMD Biosciences). 5μM of the broad-spectrum cathepsin inhibitor, E-64 (EMD Biosciences), was added for negative controls. Cultures were incubated for 8 hours, washed, and imaged using a Nikon Ti-E fluorescent microscope. Fluorescence was quantified by averaging pixel intensity across images of a given area using ImageJ.

3.2.6 Phosphorylated kinase analysis with Bioplex

HAEC or co-culture lysates were prepared according to Bioplex instructions (BioRad), and beads conjugated with antibodies for phosphorylated Akt, extracellular signal-regulated kinases 1 and 2 (ERK 1/2), c-Jun NH2-terminal kinase (JNK), and c-Jun (BioRad) were incubated overnight, followed by labeling with biotinylated secondary antibodies for 1 hour, then with avidin/streptavidin conjugated with phycoerythrin. Phosphorylated kinase levels were measured using a BioPlex 200 System (BioRad).

3.2.7 Statistical Analysis

Each experimental condition was repeated with a minimum of three biological replicates and each data point is presented as the mean value and standard error of the mean. Representative images are shown. Unpaired student t-tests were used to determine statistical significance ($*p<0.05$) between most experimental groups.

3.3 Results

3.3.1 TNFα, but not heme or hypoxia, increase monocyte adhesion to endothelial cells

One of the initiating factors of arterial remodeling is the activation of vascular endothelial cells to promote monocyte adhesion. In sickle cell disease, vaso-occlusive crises create regions of local hypoxia, and increased levels of TNFα and heme are known to induce endothelial dysfunction. Therefore, we sought to determine how TNFα, heme, and oxygen levels independently and synergistically induce monocyte adhesion to endothelial cells. Co-cultures of human aortic endothelial cells (HAECs) and THP-1 monocytes were generated as previously described. TNFα induced 2.5-3 times more monocytes to adhere to endothelial cell cultures ($p\leq0.05$). Interestingly, oxygen levels had no significant effect on monocyte adhesion (Fig 3-1). Furthermore, heme did not significantly increase monocyte adhesion, but instead reduced the potency of TNFα-induced monocyte adhesion, although not significantly suggesting that

heme may interfere with ECs ability to respond to inflammatory cytokines (Fig 3-2).

3.3.2 TNFα and monocyte adhesion synergistically induce cathepsins K and V

To determine how TNFα and heme,individually and cooperatively, regulate cathepsin activity in large artery endothelial cells, we co-cultured human aortic endothelial cells (HAECs) with and without TNFα and heme, as previously described. TNFα induced cathepsin K activity (37kDa) in all conditions regardless or oxygen level and increased cathepsin V activity (35kDa). Interestingly, heme appeared to slightly decrease the induction of cathepsin K in normoxic conditions but intensified its activity in hypoxic conditions (Fig 3-3)

Furthermore, TNFα-stimulated active cathepsin K (37 kDa) in HAECs and HAEC/-monocyte co-cultures, and also increased cathepsin V expression and activity (35 kDa) by two-fold (Fig 3-4A; n=3, $p<0.05$). THP-1 monocytes alone did not stimulate cathepsin K activity, but co-culture with endothelial cells stimulated a 50% increase in cathepsin V activity (Fig 3-4A lane 3). TNFα and co-culturing with THP-1 monocytes stimulated a 460% increase in cathepsin V active enzyme compared to HAEC controls (Fig 3-4A lane 6; n=3, $p<0.05$).

In order to ascertain if the increased active cathepsin observed in the co-cultures was mediated by direct monocyte-endothelial cell contacts, paracrine factors, or some combination of both, we implemented a transwell culture system permitting exchange of soluble factors between the cell types, while being physically separated by a 0.22 μm pore size filter. Indirect communication between monocytes and endothelial cells failed to increase cathepsin V activity as high as direct contact cultures; additionally, there was no detectable cathepsin K activity without TNFα stimulation (Fig 3-4B).

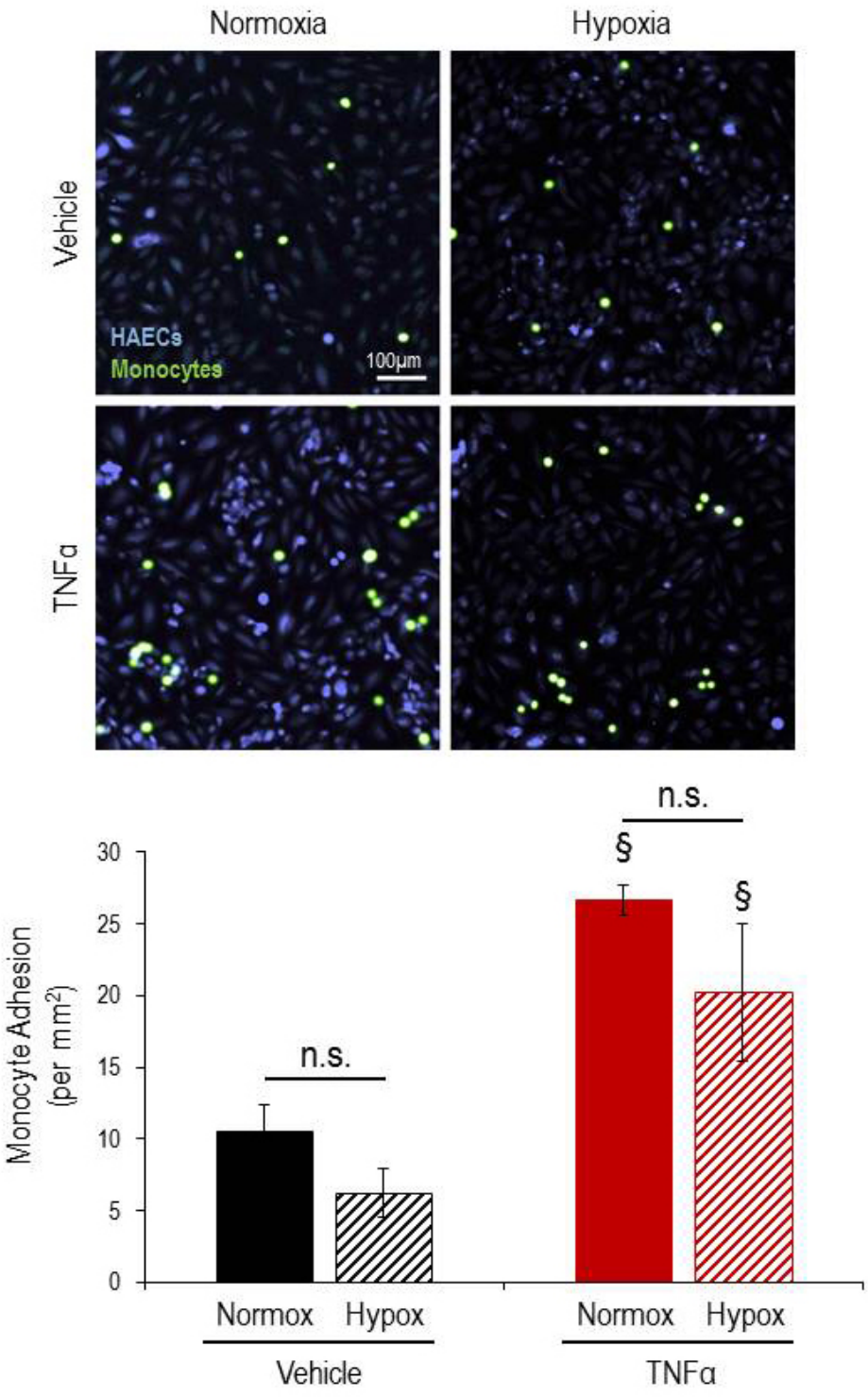


Figure 3-1: TNFα induces increased monocyte adhesion independent of environmental oxygen Confluent HAEC cultures were stained with 5μM of cell tracker red prior to stimulated with or without 10ng/mL TNFα. Cultures were then maintained in either normoxic (20% O_2) or hypoxic (1% O_2) conditions for 4 hours, after which cell tracker blue labeled monocytes were added. TNFα induced significantly higher levels of monocyte adhesion to HAECs, but oxygen level had no significant effect compared to condition matched controls. Images were pseudocolored for enhanced contrast. (n=6, § $p<0.05$, SEM bars shown.)

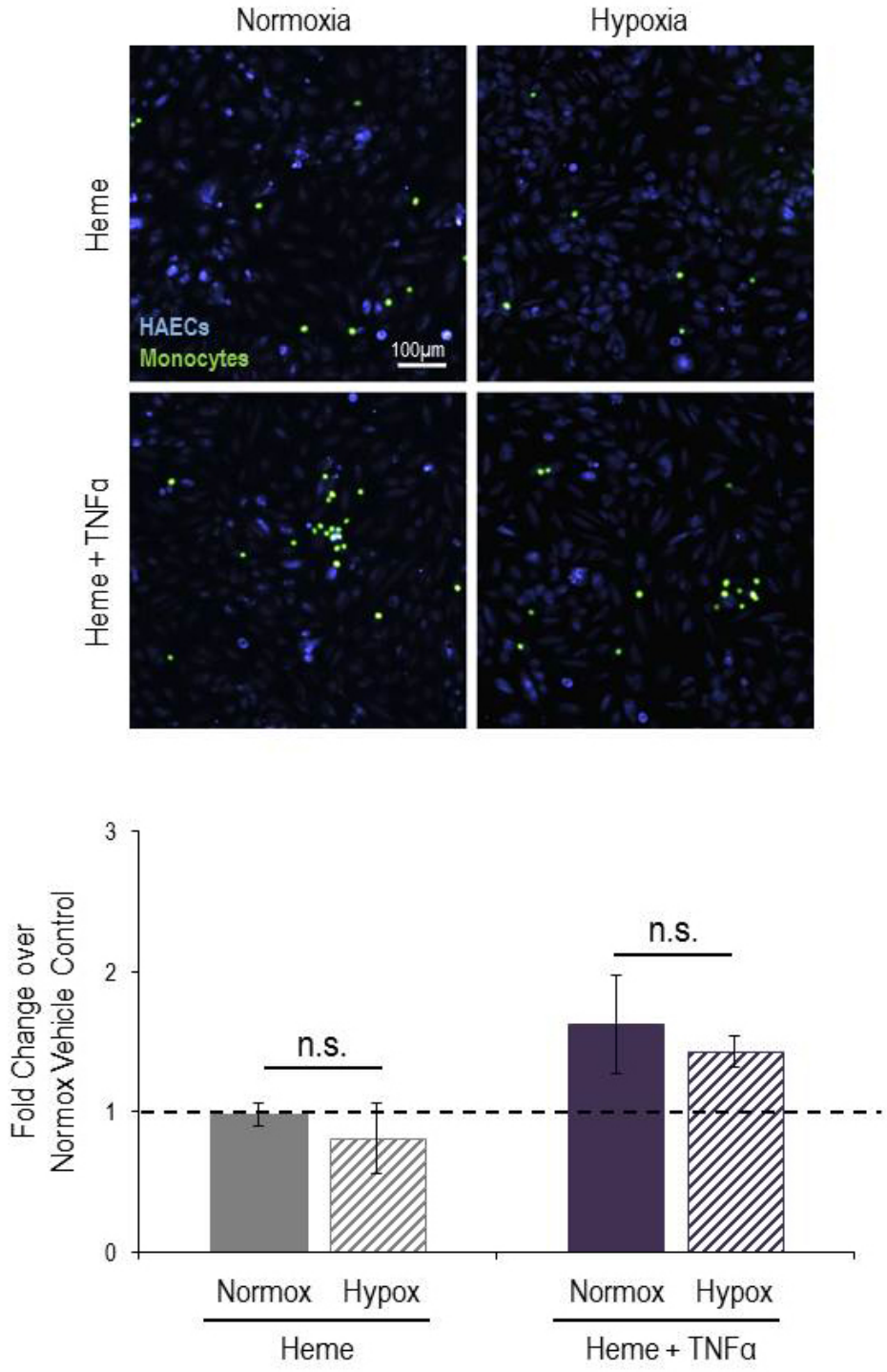


Figure 3-2: Heme does not significantly alter monocyte adhesion to large artery endothelial cells. Confluent HAECs were stained with 5μM cell tracker red stimulated and stimulated with 2.5μM heme; select cultures were simultaneously stimulated with 10ng/mL TNFα for 4 hours in either normoxic or hypoxic conditions prior to monocyte co-culture. Neither heme nor heme in combination with TNFα significantly increased monocyte adhesion regardless of oxygen level. (n=6, § $p<0.05$, SEM bars shown.)

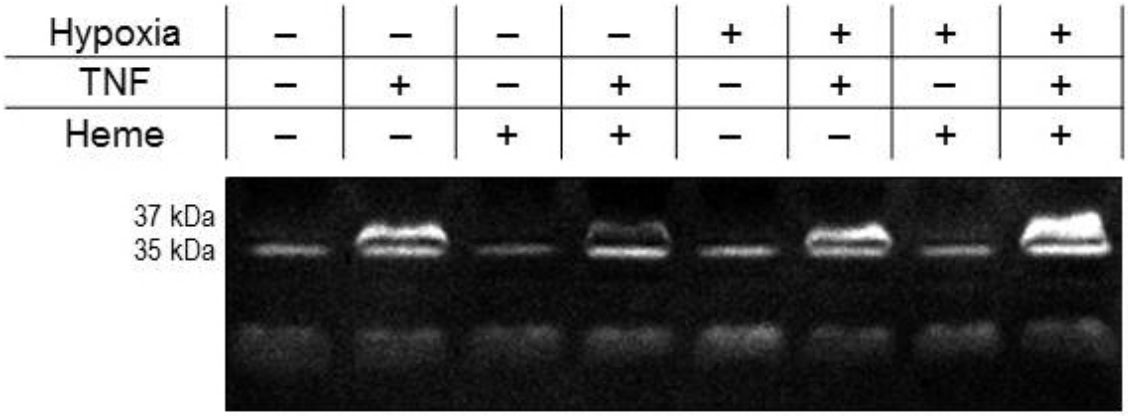


Figure 3-3: TNFα induces cathepsin K activity in large artery endothelial cells. Cell lysates were obtained from endothelial cell cultures conditioned with 10ng/mL TNFα, 2.5μM heme, or both in either normoxic (20% O_2) or hypoxic (1% O_2). Cathepsin activity as assayed using multiplex gelatin zymography.

3.3.3 TNFα turns on cathepsin K in endothelial cells

To confirm the identity of the TNFα-dependent, 37kDa active band as cathepsin K, HAECs were transfected with CMVSport6 plasmid with cathepsin K gene to drive constitutive overexpression. We achieved 25% transfection efficiency as estimated from parallel transfections with GFP vector with same concentration and protocol (data not shown). Lysates from transfected HAECs were loaded for zymography in the same gel as lysates from HAECs stimulated with TNFα or vehicle, and results are shown in figure 3-5. Transfected HAECs displayed an active band at the same electrophoretic migration distance as that of HAECs stimulated with TNFα, and with greater intensity than control cells confirming the 37kDa band as cathepsin K (Fig 3-5A). Further confirmation was achieved with an exclusionary cathepsin zymography modification; we previously demonstrated that lowering the pH from 6 to 4 during overnight incubation selects for cathepsin V activity and reduces the cathepsin K signal [147]. When incubated at pH 4, the upper 37 kDa band intensity diminished in the TNFα-stimulated samples, but cathepsin V (35 kDa) signal remained detectable

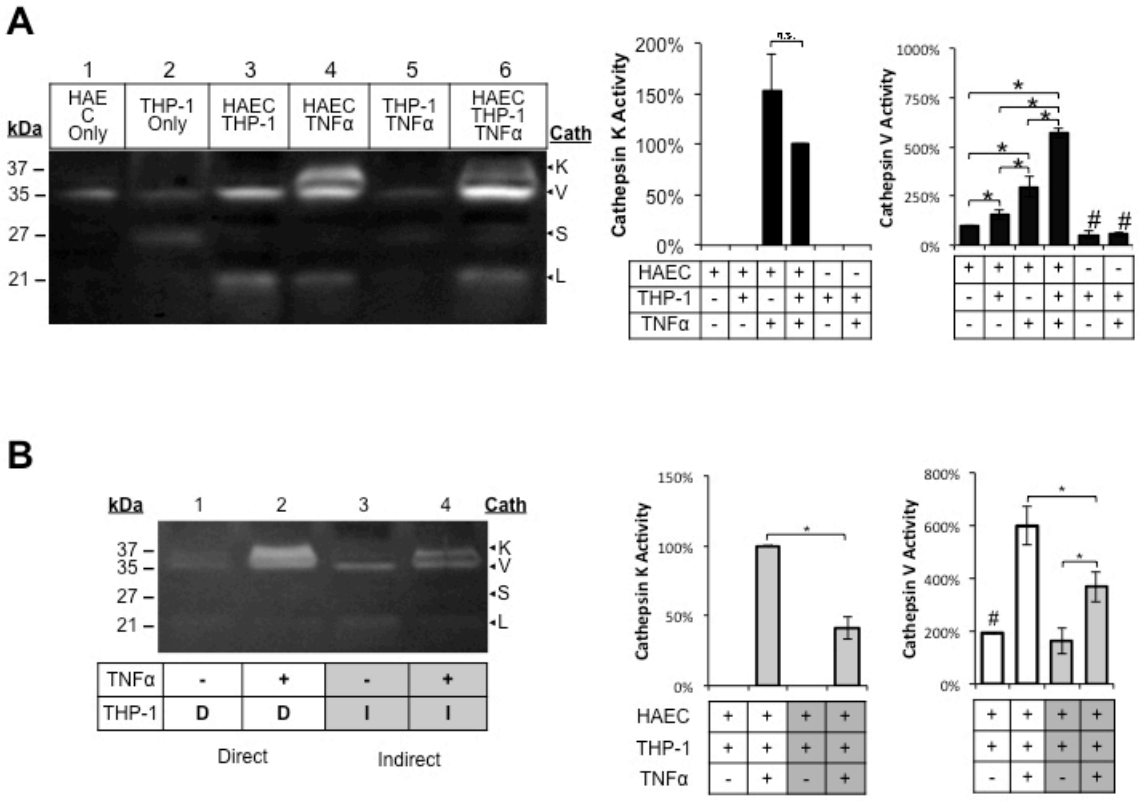


Figure 3-4: TNFα and direct monocyte adhesion induced cathepsin K and V activities in endothelial cell-monocytes co-cultures. Endothelial cells, THP-1 monocytes, and co-cultures were conditioned with 10 ng/mL TNFα. Monocytes were allowed to interact either (A) directly (indicated by "D"), or (B) indirectly, suspended above in a transwell insert with a 0.2μm pore size (indicated by "I"). (A) Cell lysates were collected and loaded for cathepsin zymography. Cathepsin K active enzyme bands were quantified with densitometry and normalized to HAEC, THP-1, TNFα samples, and cathepsin V active enzyme bands were normalized to unstimulated endothelial cell controls (n=7, *$p<0.05$, # represents significant difference from EC control, SEM bars shown). (B) Lysates from transwell cultures were also collected and loaded for zymography and active enzyme quantified with densitometry (n=3, *$p<0.05$, SEM bars shown).

under both conditions (Fig 3-5) confirming the upper band as cathepsin K.

Quenched, fluorescent synthetic substrates are commonly used to quantify the activity of cathepsin family members in cells and in vitro studies [76, 119, 147], and we used this method to identify TNFα stimulated cathepsin K activity in situ as increased fluorescence captured by microscopy. After HAECs were stimulated with TNFα, culture media was replaced with zymography assay buffer containing the cathepsin K cleavable substrate Z-GPR-MβNA (5 μM), and fluorescent images were captured. To select for the cathepsin K activity among other proteases that can cleave this substrate, parallel cultures were inhibited with 5 μM E-64 to block all cathepsin activity or with a protease inhibitor cocktail (10 μM CA-074, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM EDTA to inhibit cathepsin B, serine proteases, and matrix metalloproteinases, respectively) thereby identifying the residual activity as cathepsin K. TNFα stimulation increased total fluorescent intensity, and more importantly, the fluorescence due to cathepsin K seen after incubation with the protease inhibitor cocktail (Fig 3-5C). E-64 incubation significantly reduced fluorescent intensity as expected, as shown in the picture and indicated by the dashed line on the graph (Fig 3-5C,D).

3.3.4 TNFα stimulation and monocyte interactions with endothelial cells increased JNK and Akt phosphorylation

Next, the intracellular signal cascades initiated by TNFα and THP-1 monocyte adhesion, which appeared to have increased cathepsin K and V activities, were investigated at baseline (0 hours), after TNFα stimulation and monocyte binding (4 hours), and six hours of co-culture (10 hours). Co-cultures were maintained for 6 hours instead of 20, to shorten the length of time between stimulation and analysis to quantify the phosphorylated kinase signal before it was quiesced. Cell lysates were analyzed for phosphorylation of Akt, ERK1/2, JNK, and c-Jun using Bioplex technology. JNK and its downstream signaling protein substrate, c-Jun showed the greatest activation

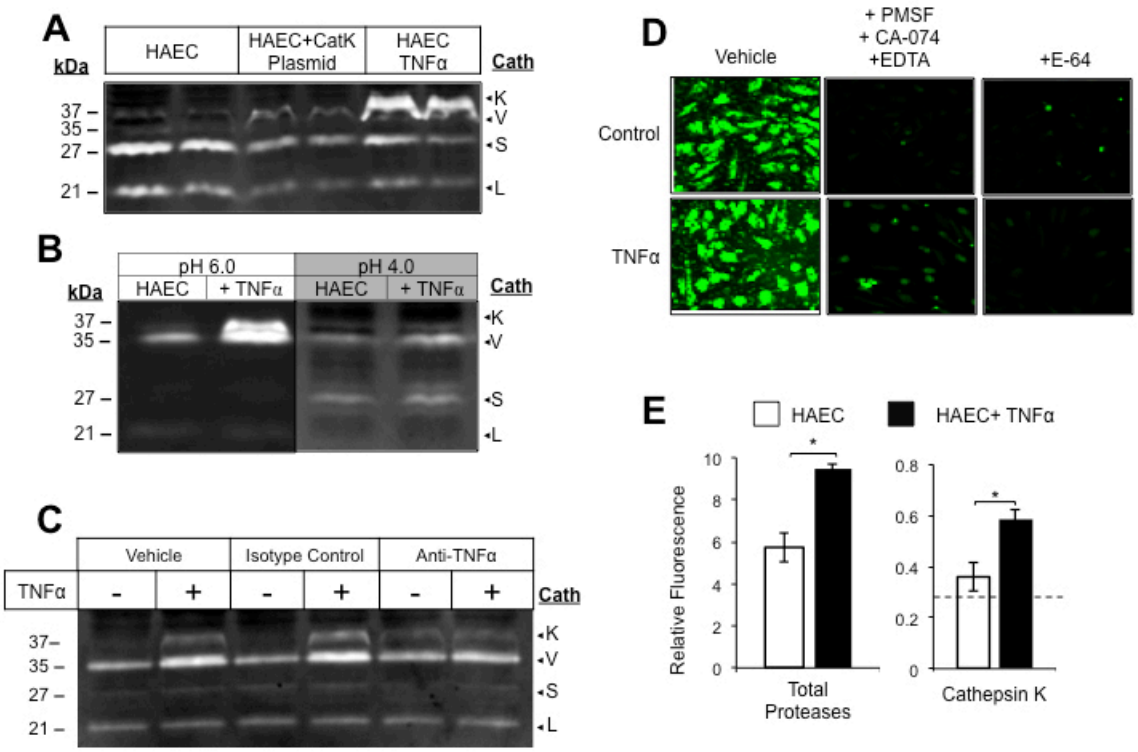


Figure 3-5: TNFα turns on cathepsin K activity in endothelial cells. (A) ECs were transfected with cathepsin K gene on pCMVSport6 to drive overexpression. Cell lysates collected from HAECs treated with and without 10 ng/mL TNFα, and ECs transfected with cathepsin K plasmid were lysed, prepared, and loaded for cathepsin zymography. (B) Cell lysates collected from HAECs treated with and without 10 ng/mL TNFα were incubated in assay buffer of pH 4 or 6 to observe the disappearance of the 37 kDa cathepsin K band at pH 4. The cathepsin K bands and TNFα stimulated bands appeared at the same molecular weight in the zymogram. (C) HAECs were stimulated with 10 ng/mL TNFα combined with either 10g/mL anti-TNFα antibody, or isotype controls. Cell lysates were collected and cathepsin activity was assessed via gelatin zymography. (D) For *in situ* zymography, endothelial cells treated with or without 10 ng/mL TNFα were incubated in zymography assay buffer containing 1mM 5-NSA and 0.5 mM Z-GPR-MβNA only or 10mM EDTA, 2mM DTT, 1mM PMSF, and 10μM CA-074 to select for cathepsin K activity, endothelial cells were also treated with 5μM E-64 to block all cathepsin activity. (E) Fluorescent images of cultures were taken and mean fluorescence intensity for total fluorescent signal and cathepsin K specific cathepsin activity was quantified.

in response to TNFα stimulation by 2.8 and 5.3 fold, respectively (Fig 3-6, n=3, p<0.01). Akt phosphorylation was significantly increased by TNFα stimulation and monocyte binding (Fig 3-6, n=3, p<0.01). There were no changes in ERK 1/2 phosphorylation in any condition for all time points measured (Fig 3-6).

JNK inhibition significantly decreased TNFα and THP-1 monocyte induced cathepsin K and V activities. Since TNFα stimulation of HAECs increased cathepsin K and V activities, and JNK and c-Jun were highly activated in response, we next tested the hypothesis that inhibiting JNK pathway would reduce cathepsin K and V activity. Endothelial cells were incubated for 1 hour with the JNK inhibitor SP6000125 (10 μM), followed by stimulation with 10 ng/mL TNFα or vehicle for 4 hours, and co-culture with THP-1 monocytes. Inhibition of JNK significantly reduced cathepsin K active enzyme by 49% in HAEC cultures stimulated with TNFα and by 39% in co-cultures stimulated with TNFα (Fig 3-7; n=3, p<0.05). In the absence of TNFα stimulation, there was no detectable cathepsin K activity. A similar effect was observed for cathepsin V; JNK inhibition reduced TNFα stimulated active cathepsin V by 60% (n=3, p<0.005) in HAECs, by 27% in co-cultures (n=3, p<0.005), and by 81% in TNFα stimulated co-cultures (n=3, p<0.001) (Fig 3-7).

3.4 Discussion

Increased cathepsin activity has been linked to tissue destructive mechanisms in the cardiovascular system including atherosclerotic elastic lamina degradation [129, 111], stent restenosis [23, 42], abdominal aortic aneurysm formation [1], and heart valve remodeling under hypertensive conditions [110]. The identification of TNFα and monocyte adhesion as both separate and partnering mediators of cathepsins K and V activation in endothelial cells via JNK signaling provides new insight into the initiation of proteolytic remodeling in cardiovascular diseases. While the progression of arterial remodeling in atherosclerosis is well described, little is known of the initial

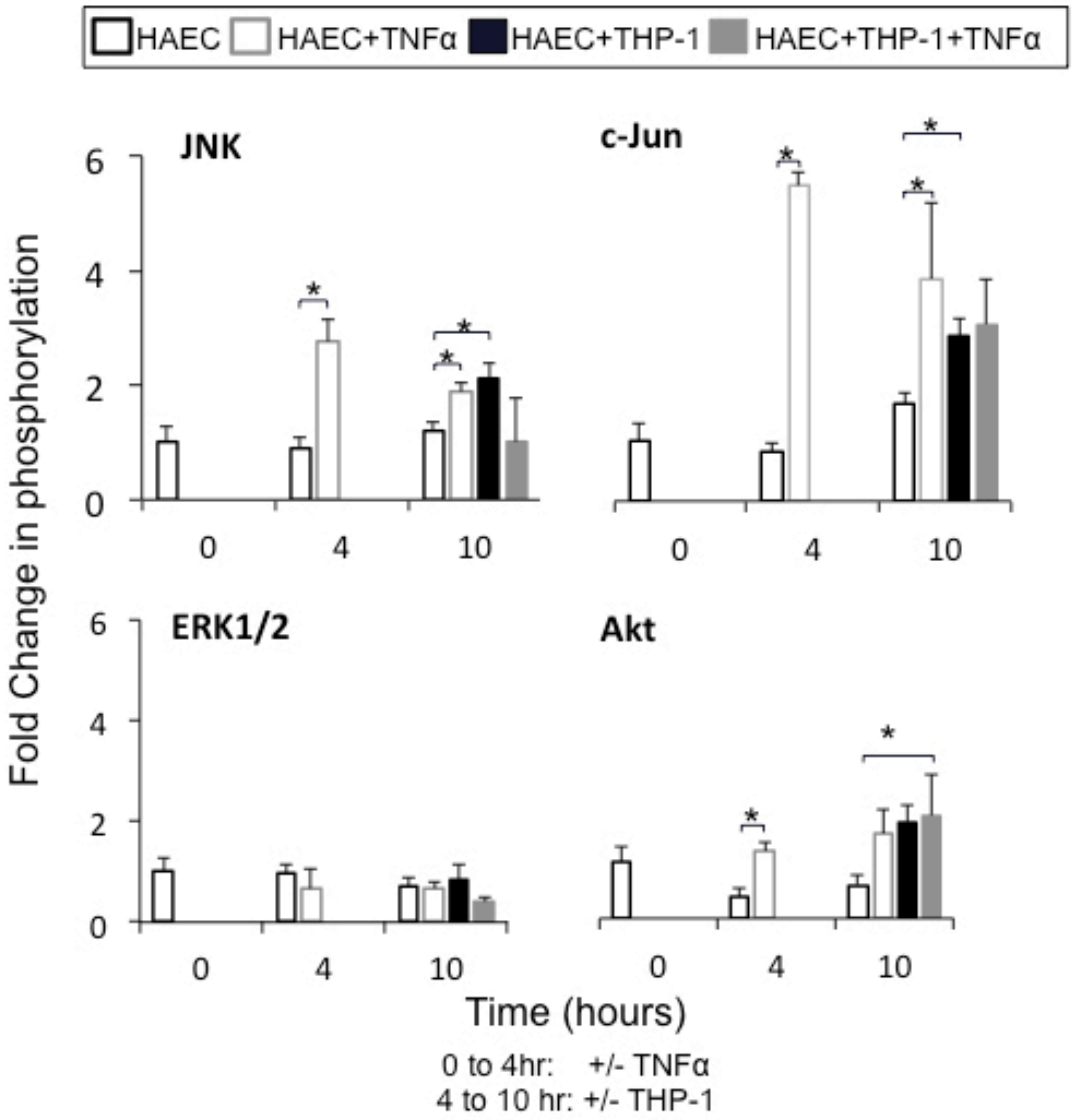


Figure 3-6: TNFα and monocyte interactions increase JNK and Akt phosphorylation. Confluent HAECs and co-cultures were pre-conditioned with 10ng/mL TNFα prior to monocyte adhesion as described earlier. HAEC and co-culture cell lysates were collected for kinase analysis using the BioPlex 200^{TM} machine that uses Luminex technology. Kinase lysates were collected prior TNFα stimulation (0 hour), 4 hours post stimulation (4 hours), and then after another 6 hours of co-culture with monocytes (10 hours). Levels of phosphorylated (A) ERK1/2, Akt, JNK, and c-Jun were measured and phosphorylated protein signal was normalized to unstimulated EC control (n=3, *p<0.05, SEM bars shown).

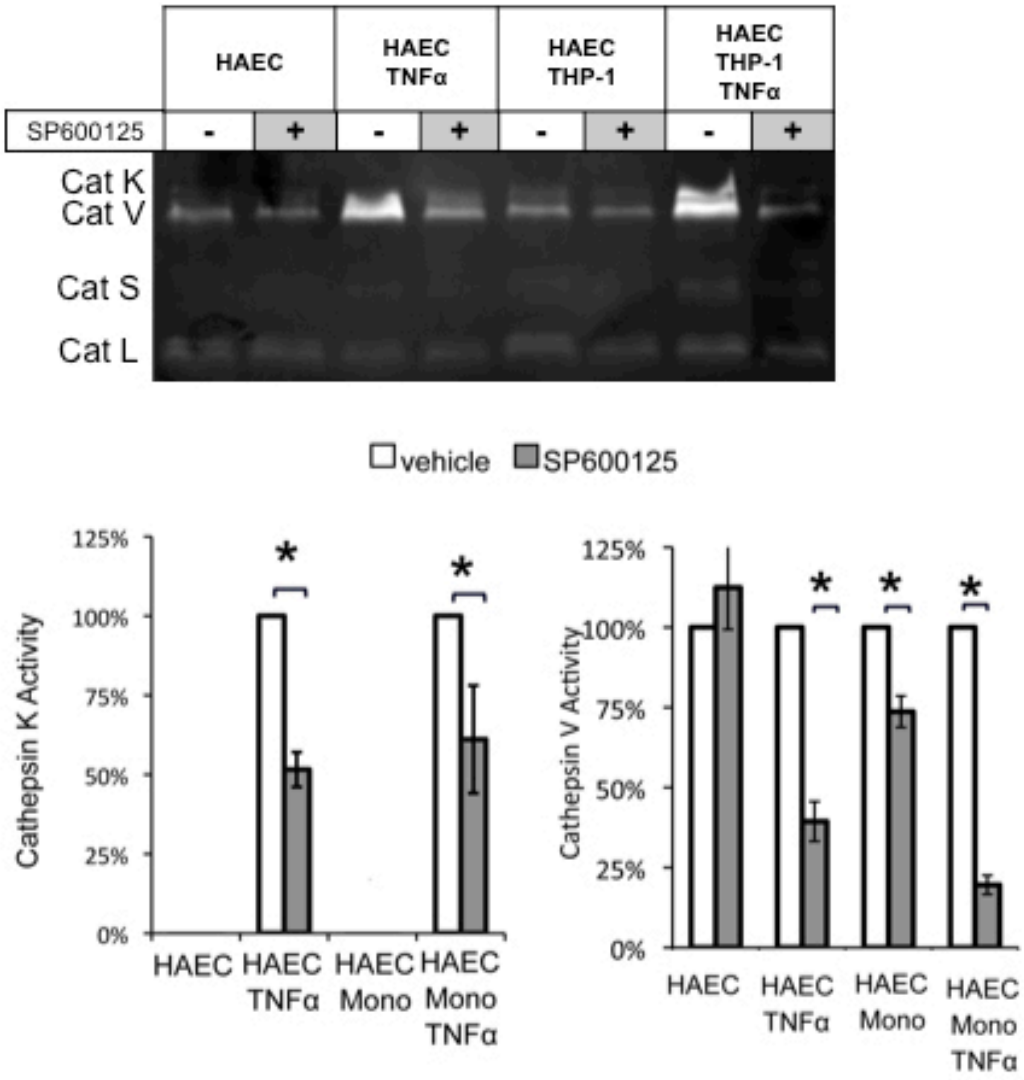


Figure 3-7: Cathepsins K and V activities induced by THP-1 monocytes are significantly reduced by JNK inhibition with SP6000125. (A) HAECs were incubated with or without 10μM of SP6000125 for 1 hour, followed by conditioning with TNFα or vehicle for 4 hours. THP-1 monocytes were subsequently added, non-adhered cells were removed, and co-cultures were maintained for an additional 20 hours. Cell lysates were collected analyzed via cathepsin zymography. (B) Densitometric analysis quantified cathepsin K and cathepsin V activity (n=3, *p<0.05, SEM bars shown).

degradation or breaks in elastic lamina that will later result in smooth muscle cell phenotypic switch and migration into neointimal space to initiate lesion formation. Here we propose that induction of cathepsin expression and mature, active cathepsins by monocyte binding to endothelial cells during the earliest steps participates in this initial elastin proteolysis.

Indirect contact between the two cell types increased cathepsin activity, but direct monocyte-endothelial cell contact induced even higher levels of active cathepsins K and V activity, even in the presence of TNFα, suggesting that juxtacrine communication is involved. Pro-TNFα present on monocyte plasma membranes is proteolytically cleaved to release the soluble cytokine [50]. Soluble TNFα then binds primarily to TNFR1 with low affinity for TNFR2, but membrane bound pro-TNFα has greater affinity for TNFR2 [136]. The direct contact between monocytes and endothelial cells may place the pro-TNFα on monocyte surfaces in close enough contact to ligate TNFR2 on endothelial cell surfaces, which may be a mechanism to explain the elevated induction of cathepsin activity with direct vs. indirect contact co-cultures (Fig 3-4B). Stimulation of either TNFR1 or TNFR2 pathway with soluble TNFα or pro-TNFα on monocyte surfaces may explain the differential regulation of cathepsins K and V in these results, but further studies are still needed.

The significant effect of JNK inhibition (Fig 3-7) on reducing cathepsin K and V activity in the co-cultures and after TNFα stimulation implicates JNK signaling cascade as a potentially successful target for therapeutic intervention. Although JNK inhibition has been shown to block ICAM-1 expression [25, 56], our studies did not show a reduction in monocyte adhesion after culturing with the JNK inhibitor, SP6000125 (data not shown) but did reduce cathepsin activity in response. It was shown previously that the transcription factor AP-1, comprised of the subunits c-fos and c-Jun, a target of JNK, stimulates cathepsin K promoter activity in macrophages [100], so the link shown here between JNK activation downstream of TNFα stimulation and

cathepsin K and V induction may involve AP-1 as well and further investigation of these pathways may be informative for reducing proteolysis during cardiovascular disease progression due to multiple cell types and their heterotypic interactions.

CHAPTER IV

SICKLE CELL DISEASE ACTIVATES PERIPHERAL BLOOD MONONUCLEAR CELLS TO INDUCE CATHEPSINS K AND V ACTIVITY IN ENDOTHELIAL CELLS[1]

4.1 Introduction

Sickle cell disease is a genetic disorder that causes *in vivo* polymerization of hemoglobin molecules into rigid fibers within red blood cells, deforming them in the canonically described "sickle" shape. Rigid, sickled red blood cells and the byproducts of their hemolysis cause chronic vascular damage and increase systemic levels of inflammatory cytokines, mobilized mononuclear cells [30], and pathological levels of increased monocyte adhesion to the endothelium [133, 10]. Overall, these pathological inflammatory conditions and mononuclear cell-endothelial cell interactions may contribute to intimal thickening, and lumen narrowing seen in pulmonary hypertension and stroke lesions of children; pulmonary hypertension is responsible for 20-30% of sickle cell related deaths in adult patients [85, 143] and 11% of children with sickle cell disease will suffer from a major stroke by the age of 16.

Development of vascular leasions in sickle cell disease, like those that lead to stroke in young children, is the result intima-media thickening of the arterial wall [13, 81, 88]. The arterial remodeling that is observed in sickle cell disease has many similarities to what has been seen in other cardiovascular remodeling diseases, such

[1]Modified from: Keegan, P. M., Surapaneni, S., & Platt, M. O. (2012). Sickle cell disease activates peripheral blood mononuclear cells to induce cathepsins k and v activity in endothelial cells. Anemia, 2012, 201781. doi:10.1155/2012/201781

as atherosclerosis, where mononuclear cell infiltration of the subendothelial space, degradation of the elastic lamina, and subsequent smooth muscle cell proliferation mediate lesion progression and luminal narrowing [133]. These similarities suggest that common mechanisms for arterial remodeling may exist between the well-studied, well-characterized atherosclerosis and the less understood mechanisms of sickle cell disease.

Arterial remodeling can be defined as changes in the composition of proteins, cell types, and even cell phenotypes that induce chronic effects on the structure, mechanical properties, and total health of the artery [14, 13, 88, 81]. This includes degradation of old matrix by newly activated proteases as well as synthesis and deposition of new extracellular matrix proteins. Cysteine cathepsins, one such family of proteases upregulated in arterial remodeling [14, 13, 112], belong to the papain superfamily of proteases and contain the most potent human collagenases and elastases [150]. Increased cathepsin activity has been linked to tissue destruction in the cardiovascular system with atherosclerotic elastic lamina degradation [111, 129], stent restenosis [23, 42], abdominal aortic aneurysm formation[1], and heart valve remodeling under hypertensive conditions [112].

Two cathepsins in particular have gained significant interest in their role in arterial remodeling in cardiovascular disease. Cathepsin K is the most potent human collagenase yet identified[44], as well as an extremely powerful elastase [59, 26]. Additionally, cathepsin K has been shown to be highly expressed in atherosclerotic lesions where it degrades arterial collagen and sub-endothelial elastic lamina [129, 111]. Cathepsin V is the most powerful mammalian elastase yet identified, and is expressed in human monocyte-derived macrophages [150]. Studies have shown that the human cathepsin V homolog, murine cathepsin L [21, 137], significantly contributes to cardiovascular disease in mouse models [149, 112]. Neither of these two enzymes have been linked to sickle cell disease induced vascular wall remodeling and pathology.

In this study, we evaluated the potential involvement of cathepsin-mediated arterial remodeling in sickle cell disease by studying the effects of TNFα stimulation and adhesion of mononuclear cells isolated from individuals homozygous for the sickle mutation on endothelial cell expression of activated cathepsins K and V. We employed a novel, multiplex cathepsin zymography technique to simultaneously quantify the active forms of cathepsins K, L, S, and V in response to the different stimulation and co-culture conditions [28]. Furthermore, we investigated the phosphorylation of key kinases to identify intracellular signaling cascades linking TNFα stimulation and mononuclear cell binding to increased levels of active cathepsins K and V as a proposed model for the unique and accelerated tissue remodeling observed in arteries of children and adults living with sickle cell disease.

4.2 Methods

4.2.1 Ethical Statement

All protocols were reviewed and approved by the Georgia Institute of Technology Institutional Review Board, and informed consent was received from all participants. In the case of minors, assent was provided by parents/guardians.

4.2.2 Culture of Primary Human Aortic Endothelial Cells

Human aortic endothelial cells (HAECs) (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% endothelial cell growth serum (ECGS). Cells were maintained with 5% CO2 at 37°C.

4.2.3 TNFα ELISA

Whole blood samples were allowed to coagulate for 6 hours, followed by centrifugation at 900g for 30 minutes to remove platelets and cells. The supernatant was collected and TNFα levels were quantified using an enzyme-linked immunosorbent

assay (ELISA) specific for soluble, human TNFα (R&D Biosystems). Absorbance values were recorded using Synergy 4TM (Biotek) at 450nm with correction readings at 540nm. Quantification of TNFα protein levels was calculated by generating a four parameter logistic standard curve using Gen5^{TM} software (Biotek).

4.2.4 Peripheral Blood Mononuclear Cell Isolation

Whole blood samples were obtained from males and females homozygous for sickle (SS) or normal (AA) hemoglobin; patients on hydroxyurea, chronic transfusion, or that had experienced a recent crisis were excluded from this study. Whole blood samples were centrifuged against a Ficoll-Paque density gradient (density: 1.077g/mL; GE Healthcare) for 30 minutes at 2450 rpm to separate the buffy coat layer. After centrifugation, peripheral blood mononuclear cells (PBMCs) were aspirated, washed in PBS, and pelleted by centrifugation for 10 minutes. The isolated cells were then washed with a red blood cell lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA) for seven minutes to remove any contaminating RBCs. Cell number and viability were determined using a Vi-Cell (Beckman Coulter).

4.2.5 PBMC Adhesion Assay

HAECs were preconditioned in normal growth media in the presence or absence of 10 ng/mL recombinant human TNFα (Invitrogen) and cultured for 4 hours prior to the addition of 500,000 PBMCs/ml. Isolated PBMCs were allowed to adhere for 45 minutes prior to washing three times with PBS, and then co-cultures were maintained for an additional 20 hours. For JNK inhibition studies, endothelial cells were preconditioned with 10 μg/mL of SP600125 (EMD Biosciences) for one hour prior to addition of media containing vehicle, 10 ng/mL TNFα, and/or 10 μg/mL of SP600125.

4.2.6 Phosphorylated Kinase Screening

Cell lysates were prepared per BioPlex Suspension Array SystemTM instructions (BioRad). Lysates were incubated overnight with fluorescently labeled beads specific for the phosphorylated forms of Akt (Ser473), extracellular signal-regulated kinases 1 and 2 (Thr202/Tyr204, Thr185/Tyr187), c-Jun NH2-terminal kinase (JNK) (Thr 183 /Tyr 185), and c-Jun (Ser63) (BioRad). The samples were then washed and incubated with kinase-specific, biotinylated antibodies for 2 hours, followed by treatment with avidin/streptavidin tagged with phycoerythrin. Phosphorylated kinase levels were measured using a BioPlex 200 System (BioRad).

4.2.7 Multiplex Cathepsin Zymography

Cathepsin zymography was performed as described previously [78]. Determination of cathepsin V band required incubation in acetate buffer, pH 4 [147]. Gels were imaged using an ImageQuant 4010 system (GE Healthcare). Images were inverted in Adobe Photoshop and densitometry was performed using Scion Image.

4.2.8 Statistical Analysis

Each experimental condition was repeated with a minimum of three biological replicates and each data point is presented as the mean value and standard error of the mean. Representative images are shown. Unpaired student t-tests were used to determine statistical significance ($*p<0.05$) between most experimental groups.

4.3 Results

4.3.1 Sickle cell disease preconditions circulating PBMCs to induce cathepsin K activity

Whole blood samples were obtained from donors homozygous for normal (AA) or sickle (SS) hemoglobin. First, an ELISA was run to quantify blood serum levels of TNFα. SS donors had 5.43 $\pm$ 2.3 pg/ml of TNFα compared to 0.3 $\pm$ 0.3 pg/ml of

TNFα in AA controls (n=3 p<0.05), an almost 20-fold increase (Fig 4-1A). TNFα stimulation of endothelial cells increased the adhesion of AA PBMCs, compared to unstimulated EC cultures (Fig 4-1B); however, the number of adhered SS PBMCs was 100 times higher than TNFα stimulated AA PBMC co-cultures (Fig 4-1B; n=3, p¡0.001). Cells were cultured together for an additional 20 hours for cathepsin induction, prior to lysing, collection, and multiplex cathepsin zymography. SS PBMCs significantly increased levels of active cathepsins K and V when co-cultured with endothelial cells, and without exogenous TNFα stimulation (Fig 4-1C), suggesting that the SS PBMCs were preconditioned to induce this activity. AA PBMC co-cultures in the absence of TNFα lacked detectable bands of active cathepsin K (Fig 4-1C, left lane).

4.3.2 TNFα stimulation and PBMC interactions with endothelial cells activate JNK signaling

To investigate the intracellular signaling cascades increasing the levels of active cathepsins K and V downstream of TNFα and PBMC adhesion cues, we measured phosphorylation of JNK, c-jun, Akt, and ERK1/2 using Bioplex/Luminex technology, a quantitative bead-based immunofluorescent assay that allowed measurement of all four signals in one cell extract after 24 hours of co-culture. JNK and its downstream signaling protein substrate, c-Jun showed the greatest activation in response to TNFα stimulation with or without AA or SS PBMCs (Fig 4-2A, B, n=3, p<0.01) with c-Jun activation as high as 6-fold that of the EC controls. Akt phosphorylation was significantly increased by AA PBMC binding alone even without TNFα stimulation (Fig 4-2C, n=3, p<0.01). There were no changes in ERK 1/2 phosphorylation in any condition for all time points measured (Fig 4-2D).

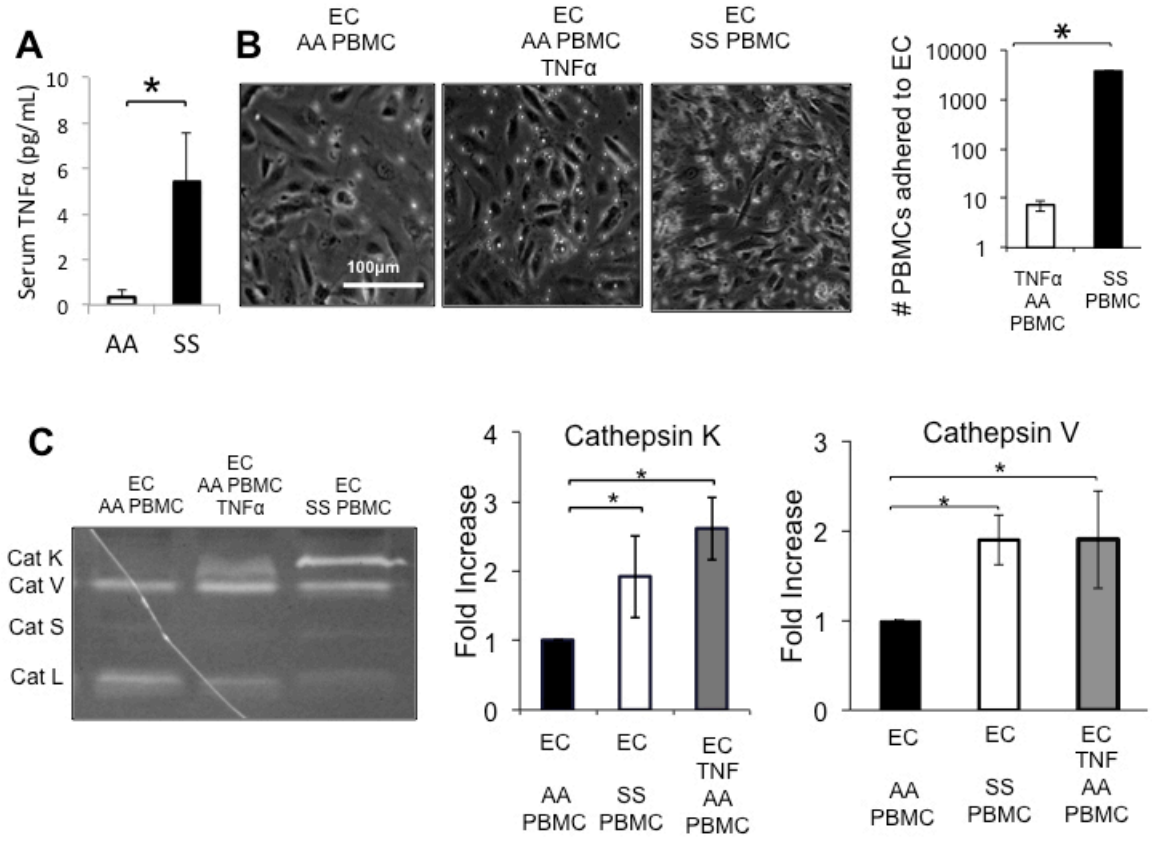


Figure 4-1: Sickle cell disease preconditions circulating peripheral blood mononuclear cells to induce cathepsin K activity. Whole blood samples were obtained from donors homozygous for the normal β-globin allele (AA) and homozygous for the sickle allele (SS). (A) Baseline serum levels of TNFα were quantified using an ELISA specific for human TNFα (n = 3, *p<0.05, SEM bars shown). (B) PBMCs were isolated via differential centrifugation through a density gradient. For co-cultures, confluent EC cultures were preconditioned with 10 ng/mL TNFα for 4 hours, prior to the addition of either AA or SS PBMCs. Nonadherent cells were washed away, and co-cultures were maintained for an additional 20 hours. Representative images of co-cultures were used for mononuclear cell adhesion counts. (C) Cells were lysed and cathepsin K activity was assessed using multiplex cathepsin zymography and quantified via densitometry (n = 10, *p<0.05).

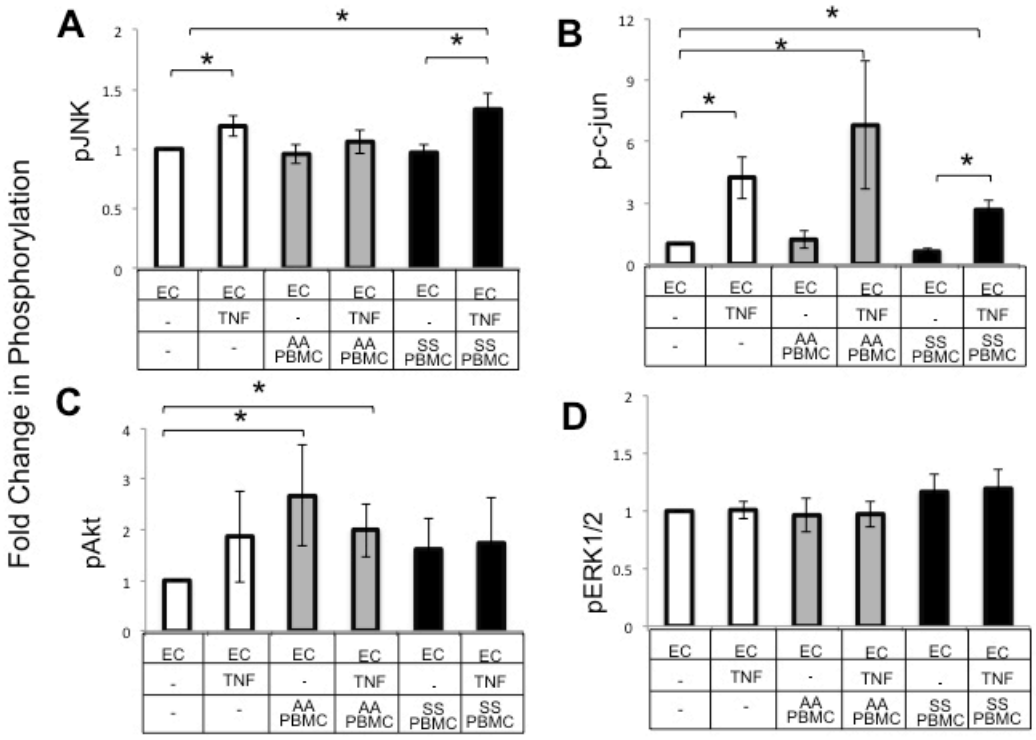


Figure 4-2: TNFα and PBMC interactions increase JNK and Akt phosphorylation. Confluent HAECs were cocultured with peripheral blood mononuclear cells isolated from AA or SS donors, and lysates were collected for kinase analysis. Levels of phosphorylated (A) JNK, (B) c-Jun, (C) Akt, and (D) ERK1/2 were measured, and phosphorylated kinase signals were normalized to unstimulated HAEC control (n = 3,*p<0.05, SEM bars shown).

4.3.3 Cathepsins K and V activities induced by sickle cell disease PBMCs were significantly reduced by JNK inhibition

Since JNK and c-jun phosphorylation were significantly upregulated, we tested if inhibiting this signal cascade would block the increase in levels of active cathepsins K and V by endothelial cells after adhesion and co-culture with SS PBMCs. HAECs were cultured with or without SP600125, a JNK inhibitor, for 1 hour prior to addition of 10 ng/mL TNFα or vehicle. AA or SS PBMCs were subsequently added, and non-adhered cells were washed away. Cell lysates were collected after 24 hours, and cathepsin activity was assessed through multiplex cathepsin zymography. SP600125 significantly reduced the upregulated cathepsin K and cathepsin V activities of unstimulated SS PBMCs when co-cultured with endothelial cells by 48% and 29%, respectively (Fig 4-3; n=5, $p<0.05$).

4.4 Discussion

Endothelial cell expression of cathepsins and increased cathepsin-mediated elastase activity are upregulated during atherosclerotic development and induced by inflammation and altered hemodynamics [11, 112, 111, 122, 129] which are both present in sickle cell disease [11], leading to our hypothesis that elevated TNFα and increased circulating mononuclear cells would stimulate increased endothelial cell cathepsin activity. This elevated activity may contribute to arterial remodeling in sickle cell disease. The findings of this study specifically implicate TNFα and mononuclear cell binding to endothelium as key mediators, and that circulating mononuclear cells in sickle cell disease are predisposed to induce cathepsin proteolytic activity.

Here, we have specifically shown that TNFα stimulation increased the expression and activity of the most potent mammalian collagenase and elastase, cathepsins K and V, respectively (Fig 4-1). Additionally, SS PBMCs significantly increased cathepsin K activity in endothelial cells in the absence of TNFα, suggesting that

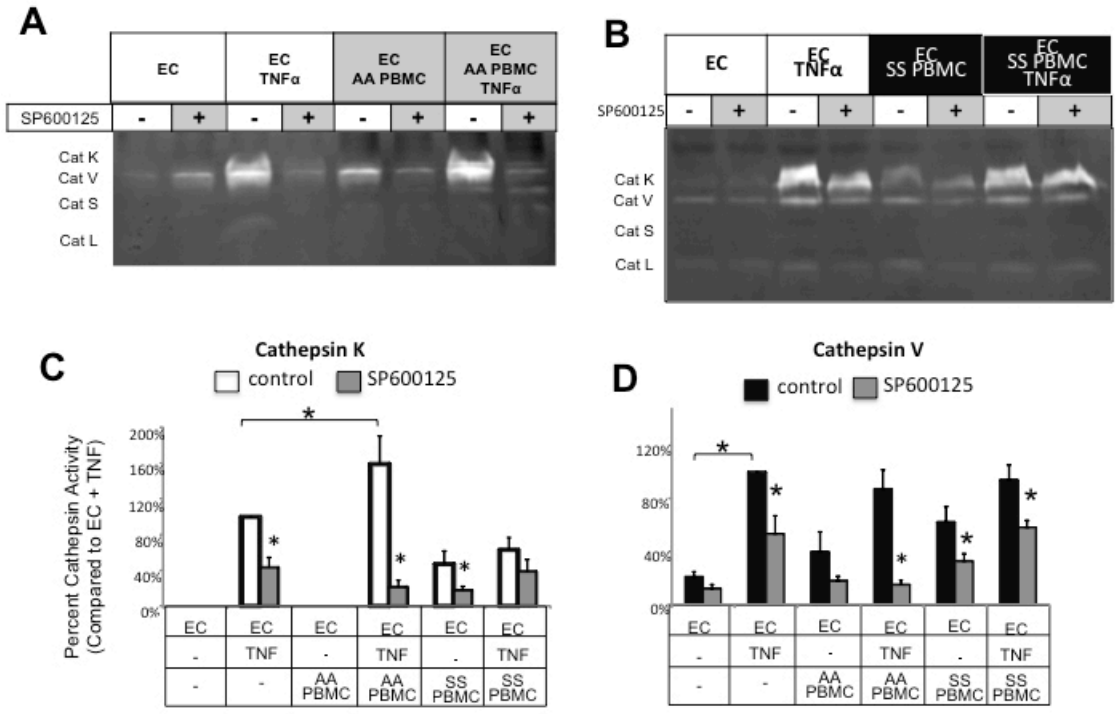


Figure 4-3: Cathepsins K and V activities induced by sickle cell disease PBMCs are significantly reduced by JNK inhibition with SP600125. HAECs were incubated with or without 10 μM of the JNK inhibitor, SP600125, 1 hour prior to TNFα stimulation, as described previously. Co-cultures with AA or SS PBMCs were maintained for an additional 20 hours. Cell lysates were collected and analyzed via multiplex cathepsin zymography. Densitometric analysis quantified active cathepsins K and cathepsin V (n = 3, *p$<$ 0.05, SEM bars shown).

they were preconditioned in the blood for adhesion to endothelium and cathepsin K induction (Fig 4-1); AA PBMCs required TNFα stimulation to reach these higher levels of cathepsin K and V (Fig 4-1). These findings are consistent with reports that circulating sickle erythrocytes increase mononuclear cell activation and adhesion to endothelial cells[153], and support our hypothesis that the blood milieu of people living with sickle cell disease predisposes circulating mononuclear cells to adhere to endothelium and promote arterial remodeling. Previous studies have already established that the circulatory environment in sickle cell disease preconditions peripheral blood mononuclear cells into a pathologically activated state, where these cells produce 139% more TNFα per cell than control mononuclear cells [14, 153]; these mechanisms may be at play here leading to increased active cathepsins K and V.

Inhibition of JNK signaling with SP600125 reduced the inflammation-induced activation of cathepsins K and V in AA and SS PBMC co-cultures with endothelium (Fig 4-3). These findings highlight the role of JNK signaling as an integration control point and as a therapeutic target to inhibit the initiation of gene and protein expression in response to inflammatory stimuli resulting in endothelial cell upregulation of cathepsins K and V protein and activity. More importantly, the predisposition of SS PBMCs to induce these effects suggests that these novel mechanisms may be occurring constantly in the vasculature of individuals with sickle cell disease. It will be important to continue these studies by quantifying cathepsin activation of SS donors with and without a history of stroke, or coupling these analyses with high transcranial doppler velocity measurements which are known to be a risk factor for stroke, in order to parse differential activation mechanisms potentially responsible for the increased risk. Such investigations may reveal novel biomarkers relevant to stroke risk prediction in pediatric patients and open new avenues for pharmaceutical therapies to prevent the arterial remodeling and luminal narrowing that cause cardiovascular

complications and death.

4.5 Conclusion

Elevated inflammatory factors and circulating mononuclear cells inherent to sickle cell disease induce pathologically high levels of cathepsins K and V activity when binding to and stimulating endothelial cells, increasing proteolytic activity that may be involved in arterial wall remodeling to increase risk of stroke and pulmonary hypertension. There is a pressing need for novel pharmaceutical targets to inhibit these activities, and from this work, we propose that JNK, cathepsin K, and cathepsin V are three new targets for inhibition to reduce pathological arterial remodeling in sickle cell disease.

CHAPTER V

NFKB AND JNK SIGNALING INTEGRATE PATHOLOGICAL BIOMECHANICAL AND INFLAMMATORY SIGNALS TO REGULATE CATHEPSIN K PROTEOLYTIC ACTIVITY IN AORTIC ENDOTHELIAL CELLS: IMPLICATIONS FOR CHRONIC VASCULOPATHY IN SICKLE CELL DISEASE.[1]

5.1 Introduction

Sickle cell disease is a genetic disorder affecting nearly 100,000 Americans and millions worldwide. Although commonly regarded as a hematological disorder characterized by the unique, sickle red blood cells, people with sickle cell disease have an 11% chance of stroke before the age of 20, with the highest risk between 2 and 5 years old [10, 2, 115]. Post-mortem examination of the cerebral vasculature of children who have suffered strokes revealed significant luminal narrowing, as well as delamination and fragmentation of the internal elastic lamina of the internal carotid and middle cerebral arteries [133, 53, 116]. Moreover, in contrast to other cardiovascular diseases in which vascular remodeling develops over decades prior to clinical presentation, arterial remodeling in children with sickle cell disease progresses at a dramatically accelerated rate. Despite the high risk and rapid formation of arterial remodeling in sickle cell disease, there currently exists no mechanistic understanding of how these

[1]Modified from: Keegan, P. M., Anbazhakan, S., Pace, B.S. & Platt, M. O. NFKB and JNK signaling integrate pathological biomechanical and inflammatory signals to regulate cathepsin K proteolytic activity in aortic endothelial cells: implications for chronic vasculopathy in sickle cell disease. *Manuscript in preparation*

vascular lesions form or progress. However, the unique inflammatory and hemodynamic circulatory environment in sickle cell disease led our group to hypothesize that pathological proteolytic remodeling may play an important role in vascular lesion development.

An often overlooked complication associated with sickle cell disease is the perpetuation of a chronic inflammatory syndrome characterized by elevated serum levels of the inflammatory cytokine tumor necrosis factor alpha (TNFα); in fact, people with sickle cell disease exhibit over 20-fold higher levels of baseline serum TNFα [67]. Additionally, people with sickle cell disease also have highly elevated numbers of circulating monocytes and increased monocyte-endothelial cell interactions throughout the vascular tree. Both elevated serum levels of TNFα and monocyte-endothelial cell interactions have been strongly correlated with the initiation and progression of many cardiovascular diseases characterized by arterial remodeling, such as atherosclerosis [71, 91]. Work by our group has shown that arterial endothelial cells respond to both elevated TNFα levels and interactions with sickle white blood cells by activating the powerful elastase and gelatinase, cathepsin K [68, 67]. Cathepsin K has become of particular interest in the context of arterial remodeling as it is the most powerful human collagenase, an extremely potent elastase [59], and has been strongly implicated in the progression of several cardiovascular diseases such as atherosclerosis [83, 80] and abdominal aneurisms [81]. In addition to responding to circulating inflammatory signals, arterial endothelial cells are sensitive to biomechanical signals, such as blood flow.

Endothelial cells are capable of monitoring and responding to hemodynamic shear stress, generated by blood flow through a vessel. Alterations in hemodynamic shear stress stimulate various cellular responses within endothelial cells, resulting in dynamic changes to the arterial environment. It is because of the strong mechanosensitive nature of endothelial cells, and their ability to affect not only the function, but

also structure, of the artery that hemodynamic shear stress represents of the critical mediators of arterial remodeling diseases [88, 60, 74]. Vessels exposed to high magnitude, unidirectional shear stress (vasoprotective) statistically are not at risk for pathological arterial remodeling [152, 49] while vessels exposed to low magnitude, oscillatory shear stress (pro-remodeling) are at significantly higher risk for arterial remodeling pathologies [73, 49]. Previous work done by our group and others have shown that cathepsin K is also shear regulated, with pro-remodeling shear stress stimulating increased cathepsin K expression and activity [27, 111].

In patients who have suffered a sickle cell related stroke, vascular lesions are commonly found in the cerebral and carotid arteries [90, 118, 2, 18, 48]. However, these arteries may spontaneously develop regions of pro-remodeling shear stress due to adhesion of red and white blood cell aggregations along the arterial wall [11] providing a second, independent, pro-cathepsin K signal to the vascular wall. Taken together, the cerebrovasculature of children with sickle cell disease represents a unique amalgam of inflammatory and biomechanical factors that, independently, are known to activate cathepsin K; however, the interplay between TNFα and shear stress regulation of cathepsin activity in large artery endothelial cells remains unclear. In this study, we developed an in vitro model to independently and combinatorially assess physiologically relevant stimuli and define a mechanistic pathway by which large artery endothelial cells simultaneously integrate TNFα stimulation and pathological hemodynamic shear stress via the JNK and NFκB pathways to regulate cathepsin expression and enzyme activity.

5.2 Methods

5.2.1 Cell Culture and Actuation of Physiological Shear Stress

Human aortic endothelial cells (HAECs) (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% endothelial cell growth supplement (ECGS) isolated from bovine cerebral tissue. Vasoprotective and pro-remodeling shear stress profiles were generated by digitizing physiological the physiological shear stress waveforms recoded by Dai et al [32, 31]. The vasoprotective waveform, characterized by high, unidirectional wall shear stress was obtained from the distal internal carotid artery as it is a representative region protected from atherogenesis; conversely, the pro-remodeling waveform was obtained from the carotid sinus due to it experiencing the highest degree of low, oscillatory shear stress and being vulneratble to pathological arterial remodeling. HAECs were grown to confluence in a 10cm dish, and stimulated with or without 10ng/mL recombinant human TNFα (Invitrogen), then placed into a cone-and-plate bioreactor, and exposed to vasoprotective or pro-remodeling shear stress for 20 hours (Appendix A; Fig 5-1). For JNK inhibition studies, confluent HAECs were stimulated with 10μg/mL of the JNK inhibitor SP6000125 (Invitrogen) for 1 hour prior to treatment with 10ng/mL TNFα. For NFκB inhibition studies, 5μM of the inhibitor Bay 11-7082 (Sigma) which selectively and irreversibly inhibits NFκB activation by blocking phosphorylation of IκBα was added to cultures for 1 hour prior to stimulation with TNFα.

5.2.2 Multiplex Cathepsin Zymography and Western Blot

Cells were lysed using zymography lysis buffer (20 nM TrisHCl [pH 7.5], 5 mM ethyleneglycoltetraacetic acid [EGTA], 150 mM NaCl, 20 mM β-glycerol phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, and 0.1% Tween 20) with 0.1% leupeptin [78, 147]. To assess cathepsin activity, cell lysates were analyzed using

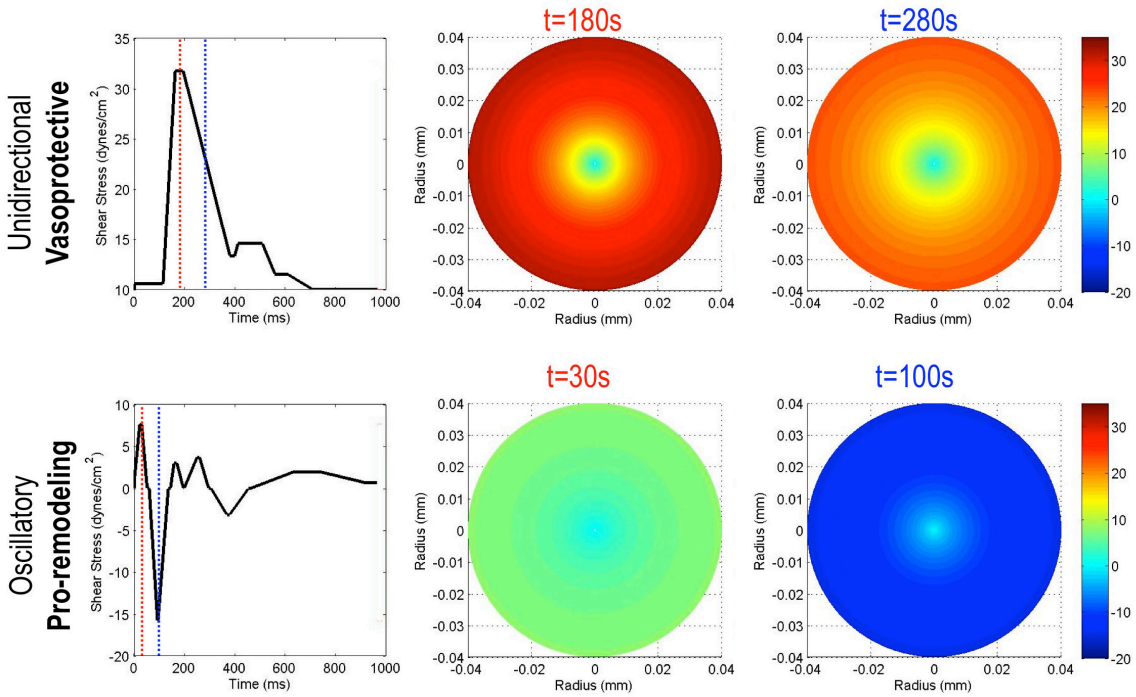


Figure 5-1: Physiological vasoprotective and pro-remodeling waveforms actuated by cone-and-plate bioreactor. Digitization of the time-dependent wall shear stress functions at the atheroprotected and atheroprone regions of the human carotid sinus as previously derived by Dai *et al.*[31]

multiplex cathepsin zymography, as described previously [78, 147, 67, 68]. Gels were imaged using an ImageQuant 4010 system (GE Healthcare). Images were inverted in Adobe Photoshop and densitometry was performed using NIH ImageJ. Western blotting was used to determine phosphorylated and total c-Jun N-terminal kinase (JNK); Cell Signaling) and c-jun primary antibodies (Cell Signaling) and secondary antibodies to be visualized with a LI-COR Odyssey scanner. Densitometry of labeled nitrocellulose membranes was performed using NIH ImageJ.

5.2.3 Chromatin Immunoprecipitation Assay for Activator Protein 1

Isolation of HAEC genomic DNA for chromatin immunoprecipitation (ChIP) was completed using the EZ-Chip system (Millipore). Briefly, after isolation of genomic DNA, and fragmentation via sonication for 5 seconds at 15% amplitude followed by a 10 second rest was repeated 22 times for a total sonication time of 110 seconds. PCR primers were synthesized based on previously identified using previously identified binding regions for activator protein 1 (AP-1) on the cathepsins K geneGelb:1997tx. The forward sequence, 5'-TCCTAACAGGAAAGGGGTAGGA-3', and the reverse sequence, 5'-AGACTGTCTTTGGTGGCAAAT-3', were analyzed using the Basic Local Alignment Search Tool (BLAST; NIH) to ensure minimal cross reactivity with other sequences prior to synthesis (Integrated DNA Technologies).

5.2.4 Quantification of cathepsin K mRNA

mRNA was isolated from shear conditioned HAEC cultures using RNeasy mini-prep (Quiagen) and reverse transcribed to cDNA using the forward primer sequence 5'-ATATGTGGGACAGGAAGAGAGTTGT-3' and the reverse primer sequence 5'-GGATCTCTCTGTACCCTCTGCATTT-3'. Quantitative real time PCR was performed and relative cathepsin K mRNA synthesis was quantified using $\Delta\Delta C_t$

5.2.5 Immunostaining

As described previously, confluent HAEC cultures were conditioned with 10ng/mL TNFα, and maintained under static, vasoprotective, or pro-remodeling shear stress for 20 hours. Cells were rinsed with PBS three times, and fixed with 4% paraformaldehyde (PFA) for 10 minutes, rinsed with PBS, permeabilized using 0.2% Triton X, and blocked with 3% BSA in PBS for 1 hour at room temperature. HAECs were incubated overnight at 4C with monoclonal rabbit anti-cathepsin K (Cell Signaling; 1:100), monoclonal rabbit anti-phosphorylated c-Jun (Cell Signaling; 1:100), or monoclonal rabbit anti-NFkB (Cell Signaling; 1:50). Cells were rinsed three times with PBS, and then incubated with Alexa Fluor 488 conjugated secondary antibodies (Invitrogen; 1:150) for 1h at room temperature. Cultures were counterstained with Alexa Fluor 568 phalloidin (10μg/mL; Invitrogen), and Hoechst (10mg/mL; Invitrogen), mounted, and cover slipped. Images were acquired using a Zeiss LSM 700-405 confocal microscope.

5.2.6 Statistical Analysis

Each experimental condition was repeated with a minimum of three biological replicates and each data point is presented as the mean value and standard error of the mean (SEM). Representative images are shown. Unpaired student t-tests were used to determine statistical significance (*$p<0.05$) between most experimental groups.

5.3 Results

5.3.1 Vasoprotective shear stress is sufficient to reduce the amount of active cathepsin K in endothelial cells

We first examined the regulation of cathepsin K in response to simultaneous integration of physiological shear stress with TNFα stimulation in large artery endothelial cells. HAECs were grown to confluence and stimulated with TNFα and simultaneously exposed to either vasoprotective or pro-remodeling shear stress. Pro-remodeling

shear stress (Fig 5-2 A, Lane 5) induced a 1.5-fold higher level of active cathepsin K compared to TNFα-stimulated cells cultured in static conditions (Fig 5-2 A, Lane 2); co-stimulation of pro-remodeling shear stress with TNFα did not significantly increase cathepsin K activity compared to pro-remodeling shear stress alone. Vasoprotective cultures stimulated with TNFα exhibited a 0.5-fold decrease in cathepsin K activity (Fig 5-2 A Lanes 4; n=3 $p<0.05$). Immunocytochemistry staining for intracellular cathepsin K in HAECs indicated that TNFα-stimulation, and pro-remodeling shear stress both increase protein expression of cathepsin K in HAECs. Conversely, vasoprotective shear stress reduced staining of intracellular cathepsin K, even in the presence of TNFα (Fig 5-2B). These findings suggest that biomechanical cues may be capable of enhancing or repressing the proteolytic response to inflammatory stimuli.

Cystatins are potent physiological inhibitors of cathepsin activity, and are ubiquitously expressed by all cell types in the body. Cystatin B primarily functions to inhibit intracellular cathepsin activity activity, and while cystatin C primarily inhibits extracellular activity, it can be taken up by cells and inhibit cathepsins along the endocytic pathway. We have so far observed that hemodynamic shear stress significantly reduces the amount of activate cathepsin K produced by arterial endothelial cells; therefore we next hypothesized that hemodynamic shear stress may regulate cathepsin activity through differential expression of cystatin B and C. Cultures were immunostained for cystatin B protein expression showed no substantive changes in response to either shear stress or TNFα stimulation, suggesting that the changes in active cathepsin K levels as a result of either vasoprotective or pro-remodeling shear stress are not likely due to increased cystatin B-cathepsin K interactions (Fig 5-3).

Additionally, we also investigated the potential for cystatin C to be shear regulated. Immunostaining of sheared HAECs indicated that pro-remodeling shear stress had markedly increased intracellular cystatin C levels compared to vasoprotective shear stress (Fig 5-4), which is in apparent opposition to what observed cathepsin

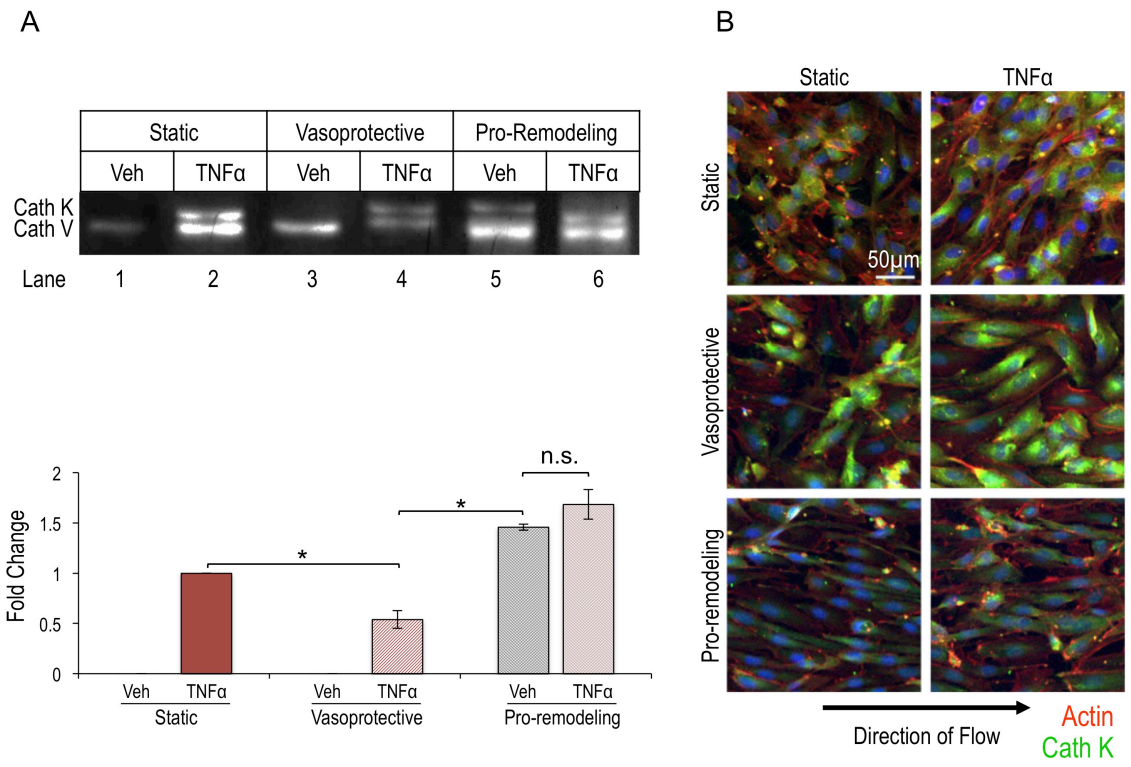


Figure 5-2: Vasoprotective shear stress reduces TNFα induced cathepsin K protein and activity. Confluent HAECs were stimulated with 10 ng/mL TNFα, and subjected to static conditions (lanes 1 & 2), vasoprotective (lanes 3 & 4), or pro-remodeling (lanes 5 & 6) shear stress for 20 hours, (A) then lysed to quantify cathepsin activity with multiplex cathepsin zymography and densitometry. Pro-remodeling shear stress alone was sufficient to induce detectable levels of cathepsin K activity (Lane 5 vs Lane 1); however, co-stimulation with TNFα did not result in a synergistic effect. Conversely, cultures maintained under vasoprotective shear stress reduced TNFα-mediated cathepsin K activity by 0.5-fold. * denotes statistical significance with $p<0.05$. (B) Alternatively, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X, and immunolabeled for cathepsin K protein (green); cultures were counterstained with phalloidin (red) for actin, and Hoechst (blue) for nuclei. Baseline cathepsin protein in HAECs was increased in response to pro-remodeling shear stress, but vasoprotective inhibited this (n=3, representative images shown). TNFα also stimulated an increase in cathepsin K activity in static and pro-remodeling conditions, but was substantially reduced vasoprotective cultures stimulated with TNFα.

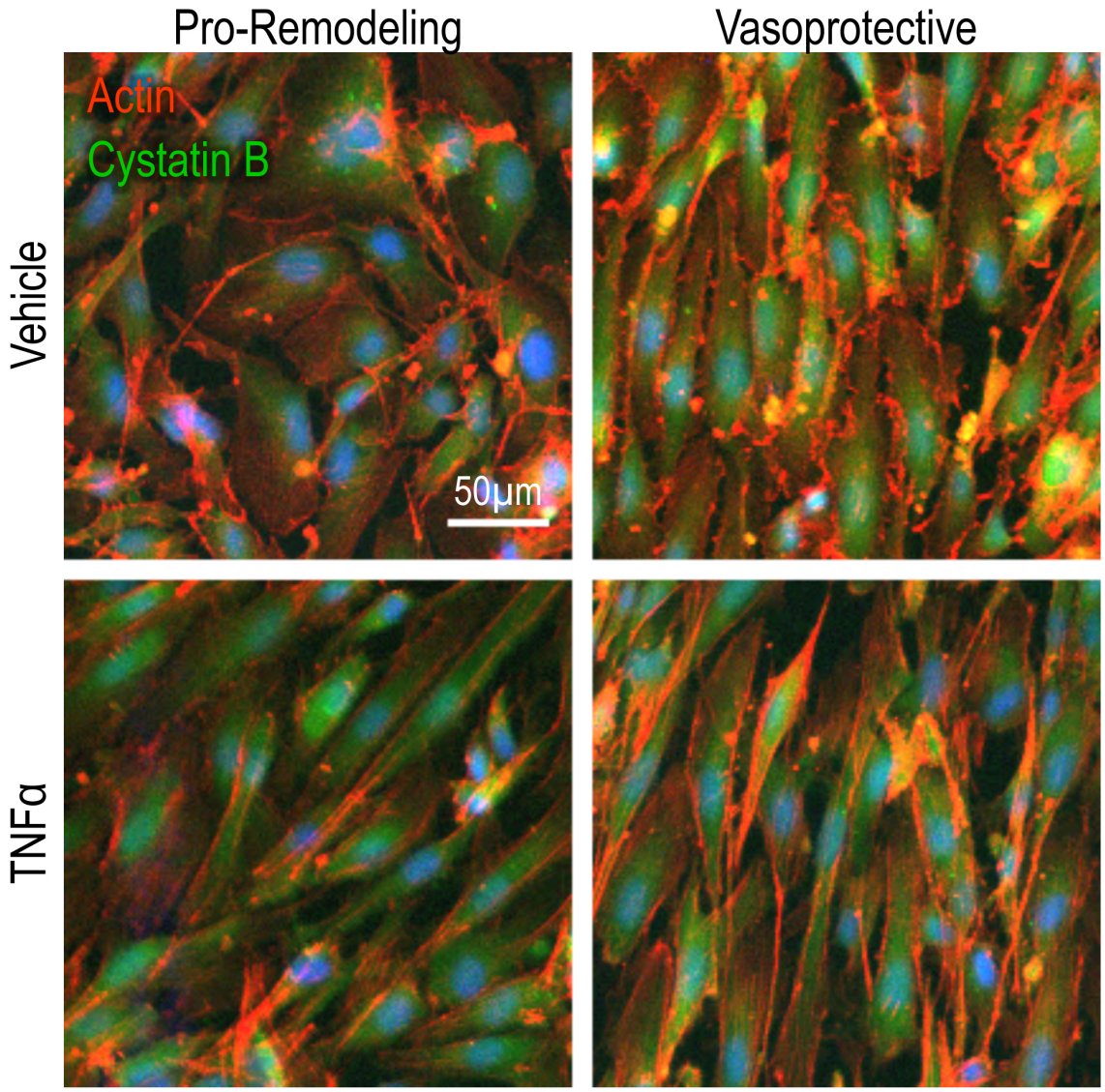


Figure 5-3: HAEC expression of cystatin B is not changed by TNFα and shear stress stimulation. Confluent HAECs were stimulated with 10ng/mL TNFα, and sheared with vasoprotective or pro-remodeling hemodynamic waveforms for 20 hours. Cultures were immunostained for expression of cystatin B (green), and counterstained for nuclear DNA (blue), and actin filaments (red) for increased visualization of the cell body.

activity in Fig 5-2. However, cystatin C is secreted by cells soon after synthesis and is likely to inhibit the intracellular cathepsin activity we assay for in this study. Upregulation of cystatin C under proremodeling shear stress likely serves to protect the extracellular environment from pathological remodeling caused by upregulated cathepsin activity.

5.3.2 Physiological Shear Stress Regulates Cathepsin K Activity Independently of JNK/c-Jun Signaling

Next, we investigated the intracellular kinase-signaling network that integrates biochemical and biomechanical stimuli. Previously, our group established that TNFα-dependent activation of cathepsin K activity in large artery endothelial cells is dependent on the JNK/c-jun signaling axis [68]. Based upon the synergistic cathepsin K activity in response to both biomechanics and TNFα, we then tested the hypothesis that physiologically relevant shear stress would also modulate JNK and c-Jun phosphorylation, leading to changes in cathepsin K expression and activity. As expected, TNFα alone resulted in 3.3-fold increase in JNK phosphorylation, and a 3.4-fold increase in c-Jun phosphorylation compared to vehicle controls (Fig 5-5; n=3, $p<0.05$). Interestingly, despite the previously established increase in cathepsin K activity, JNK and c-Jun phosphorylation were not significantly increased by pro-remodeling shear stress alone or in combination with TNFα, compared to static controls (Fig 5-5 C-D; n=3 $p>0.3$). Furthermore, while vasoprotective cultures inhibited cathepsin K activity, they did not significantly decrease phosphorylation of JNK or c-Jun, compared to controls (Fig 5-5 A-B; n=3, $p>0.8$). Together, these data confirm that the JNK/c-Jun signaling axis is involved in inflammatory cytokine induction of cathepsin K; however, the biomechanical shear stress regulation of proteolytic activity is independent of JNK/c-Jun, suggesting regulation through a secondary, independent, mechanism.

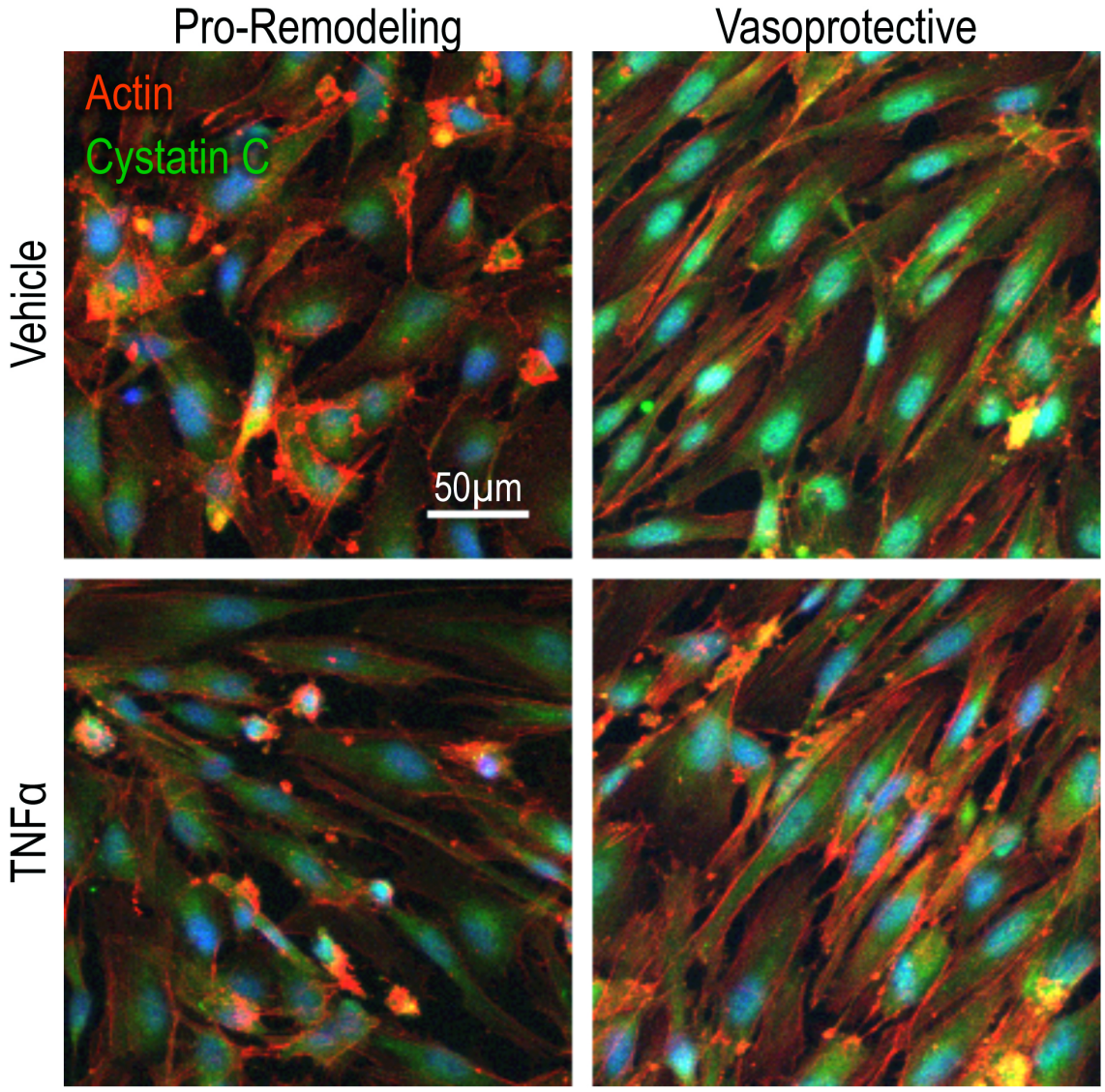


Figure 5-4: Pro-remodeling shear stress increases cystatin C expression in large artery endothelial cells. HAECs conditioned with pro-remodeling shear stress had markedly more intense cystatin C signal (green) by immunostaining, compared to cultures maintained under vasoprotective shear stress. Cultures were immunostained for expression of cystatin C (green), and counterstained for nuclear DNA (blue), and actin filaments (red) for increased visualization of the cell body.

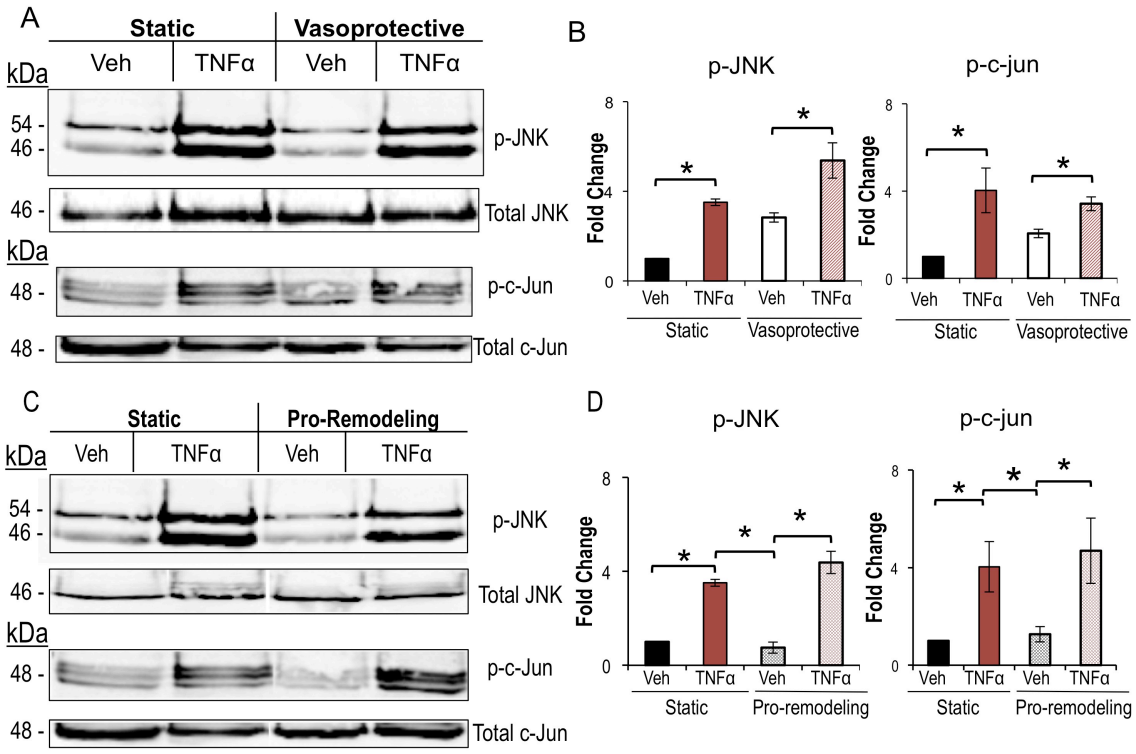


Figure 5-5: TNFα stimulates JNK/c-Jun activation under vasoprotective and pro-remodeling shear stresses. Confluent HAEC cultures stimulated with or without 10ng/mL TNFα, and maintained under static, pro-remodeling, or vasoprotective shear stress were lysed and phosphorylated and total protein levels of JNK and c-Jun were assessed using Western blot. (A, B) TNFα, alone induces a 3-fold increase in phosphorylation of JNK and c-Jun, relative to total protein levels. However, vasoprotective shear stress does not significantly alter JNK or c-Jun phosphorylation levels, regardless of TNFα stimulation. (C, D) Additionally, pro-remodeling shear stress did not alter JNK or c-Jun phosphorylation levels, relative to vehicle or TNFα static controls. * denotes $p<0.05$.

We also tested the hypothesis that hemodynamic shear stress altered transcriptional regulation of cathepsin K either by altering nuclear localization of phosphorylated c-Jun, or preventing binding of phosphorylated c-Jun to the promoter region of the cathepsin K gene. Immunostaining of sheared endothelial cells indicated that TNFα induced a substantial increase in phosphorylated c-Jun within the nucleus of HAECs compared to vehicle, as expected (Fig 5-6 A). Pro-remodeling shear stress alone also induced translocation of phosphorylated c-Jun compared to static vehicle cultures, though to a lesser extent compared to that exhibited by TNFα stimulation (Fig 5-6 A). Consistent with the Western blot results, vasoprotective shear stress did not reduce TNFα-mediated phosphorylation or nuclear translocation of c-Jun (Fig 5-6 A). Furthermore, quantification of c-Jun binding to the cathepsin K promoter region indicated that TNFα alone increased binding by 2.7-fold compared to vehicle, and was not significantly decreased by vasoprotective shear stress. Vasoprotective shear stress alone increased c-Jun binding by 8.3-fold, and TNFα co-stimulation with vasoprotective shear increased c-Jun binding to the cathepsin K promoter region by 28-fold (Fig 5-6 B; n=4, *$p<0.05$). However, despite increases in phosphorylated c-Jun binding to the promoter region of the cathepsin K gene, quantification of cathepsin K mRNA levels gene activity is significantly reduced under vasoprotective shear stress, regardless of TNFα stimulation (Fig 5-6 C). Conversely, pro-remodeling shear stress alone significantly increased cathepsin K gene activity (Fig 5-6 D), corroborating the immunostaining and zymography data. Therefore, these data confirm that, while shear stress influences both baseline and TNFα-mediated cathepsin K expression and activity, the mechanism is independent of the established TNFα-dependent JNK/c-Jun signaling axis.

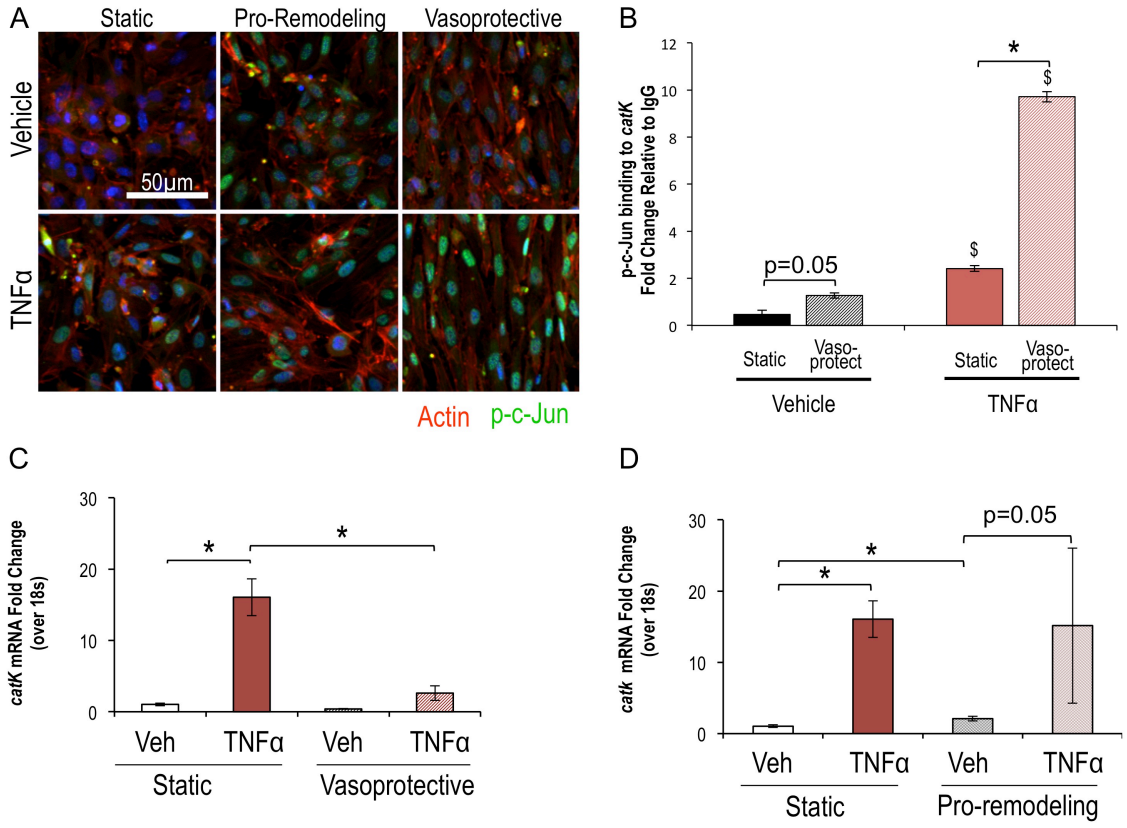


Figure 5-6: Vasoprotective shear stress does not block nuclear localization of phosphorylated c-Jun. Confluent HAECs were stimulated with 10ng/mL TNFα, and subjected to static conditions, vasoprotective, or pro-remodeling shear stress for 20 hours. (A) Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton-X, and immunolabeled for phosphorylated c-Jun (green); cultures were counterstained with phalloidin (red) for actin, and Hoechst (blue) for nuclei. No detectable levels of phosphorylated c-Jun was observed in static or vasoprotective shear stress, but pro-remodeling cultures had increased slight increase in nuclear localization of phosphorylated c-Jun. Stimulation with TNFα increased nuclear localization across all shear conditions. (B) Alternatively, cells were fixed with 4% PFA, and lysed with a 1% SDS lysis buffer to recover chromatin-protein complexes. Genomic DNA was sheared and isolated based on manufacturer's instructions. TNFα increased phosphorylated c-Jun binding to the cathepsin K promoter region by 2.7-fold under static and by 28-fold under vasoprotective shear conditions. (C-D) Additionally mRNA was isolated and cathepsin K gene expression was quantified with qRT-PCR. n=3, * denotes statistical significance with $p<0.05$.

5.3.3 Shear Stress Regulates Cathepsin K Gene Expression via NFκB Signal Transduction

To elucidate the multi-pathway cross talk in the synergistic regulation of proteolysis by biomechanical and inflammatory stimuli, we next investigated the roles of alternative downstream kinase pathways. Intracellular responses to TNFα-stimulation in endothelial cells are often divided into two canonical pathways: (1) JNK activation and downstream signaling, and (2) nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) translocation and transcriptional regulation. There is evidence to suggest that regulation and activation of NFκB is shear stress regulated, and has been implicated in arterial remodeling in vivo [135, 43, 102]. Therefore, we next investigated the potential for NFκB to function as the shear stress responsive element in endothelial cell cathepsin K activation. Immunostaining for total NFκB expression indicated that vasoprotective shear stress significantly decreased whole-cell levels of NFκB compared to pro-remodeling shear stress, regardless of TNFα stimulation (Fig 5-7). The decreased bioavailability of NFκB in response to vasoprotective shear stress was confirmed via Western blot (Fig 5-7).

To directly establish a link between NFκB and cathepsin K, we next tested the hypothesis that preventing the activation of NFκB would inhibit pro-remodeling activation of cathepsin K. Inhibition of NFκB activation reduced cathepsin K activity under pro-remodeling shear stress, both with and without TNFα, to undetectable levels (Fig 5-8 Lanes 7 and 8). Interestingly, inhibition of NFκB activation significantly reduced cathepsin K activity in pro-remodeling cultures by 0.5-fold (Fig 5-8 Lane 7 vs Lane 3), and pro-remodeling cultures co-stimulated with TNFα were reduced by 0.9-fold (Fig 5-8 Lane 8 vs Lane 4); inhibition of NFκB activation had no significant effect on TNFα-mediated induction of cathepsin K activity. Additionally, there was no significant change in total NFκB protein levels due to inhibition of NFκB activity. The findings from this data implicate shear-regulated NFκB signaling in

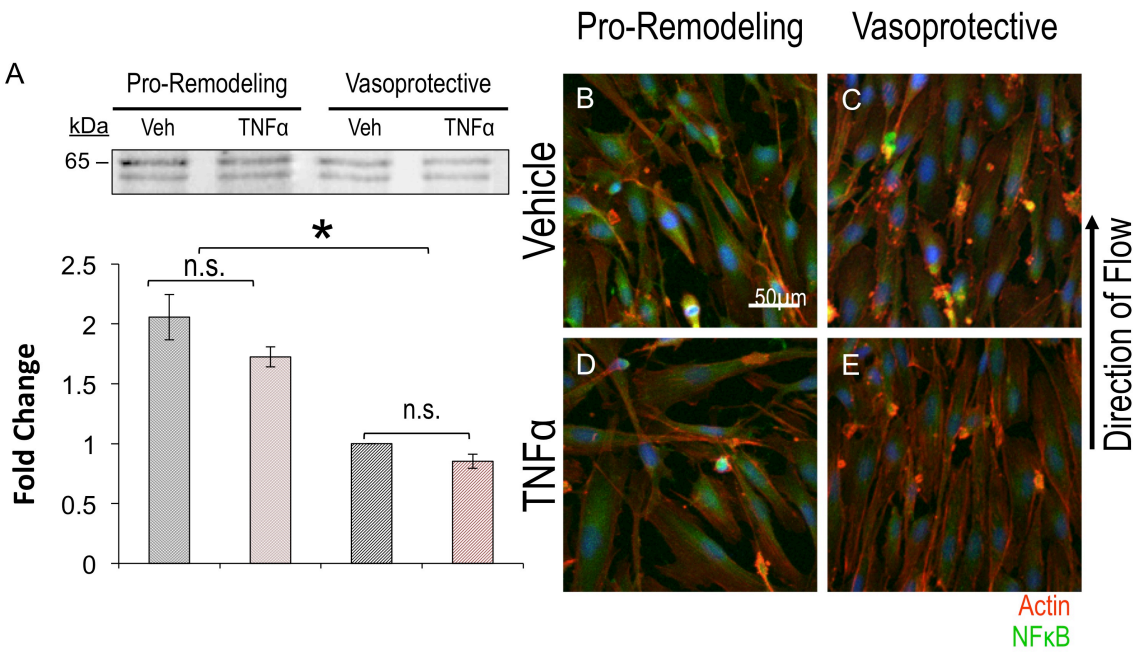


Figure 5-7: TNFα and vasoprotective shear stress decrease NFκB protein levels. Cultures were either lysed for Western blot analysis, or fixed with 4% PFA for immunohistochemistry, and stained for NFκB (green); cultures were counterstained with phalloidin (red) for actin, and Hoechst (blue) for nuclei. (A) Western blot analysis of culture lysates show a decrease in detectable, whole-cell NFκB protein levels in vasoprotective culture. (B) Pro-remodeling cultures had detectable levels of phosphorylated NFκB, but (C&E) vasoprotective cultures had substantially decreased NFκB expression.

pro-remodeling-induction of cathepsin K activity.

5.4 Discussion

In this study, we investigated how large artery endothelial cells integrate biochemical and biomechanical signals; specifically intracellular kinase signal pathway by which physiological shear stress mitigated or enhanced TNFα-stimulated cathepsin K, a powerful elastase and collagenase upregulated in other cardiovascular disease [111, 110, 80, 81]. We show here that active cathepsin K is increased in response to pro-remodeling shear stress, a physiological shear stress waveform known to induce arterial wall remodeling and atherosclerotic plaques. Additionally, cultures co-stimulated with pro-remodeling shear stress and TNFα, which we have shown previously to activate cathepsin K in endothelial cells [68, 67], increased average cathepsin K activity in endothelial cells, although the increase was not significant (Fig 5-5). Conversely, vasoprotective shear stress significantly reduced TNFα-stimulated induction of cathepsin K (Fig 5-2) independent of the JNK/c-Jun signaling pathway (Fig 5-5). These data suggest that cathepsin K activation in large artery endothelial cells is regulated by distinct signaling pathways that respond independently to biochemical (TNFα) or biomechanical (shear stress). Examination of cathepsin K gene expression indicated that TNFα induced nuclear localization of phosphorylated c-Jun and subsequent binding to the cathepsin K promoter region, regardless of shear stress conditions (Fig 5-6 A-B); however, vasoprotective shear stress did significantly decrease overall cathepsin K gene expression (Fig 5-6 C-D). Furthermore, we demonstrated that vasoprotective shear stress reduces NFκB protein levels in HAECs maintained under vasoprotective shear stress, compared to cultures maintained under pro-remodeling shear stress (Fig 5-7), and inhibition of NFκB activity significantly reduced activation of cathepsin K by pro-remodeling shear stress (Fig 5-9). Based on the findings of this study, the loss of cathepsin K activity and expression in response to TNFα in cultures

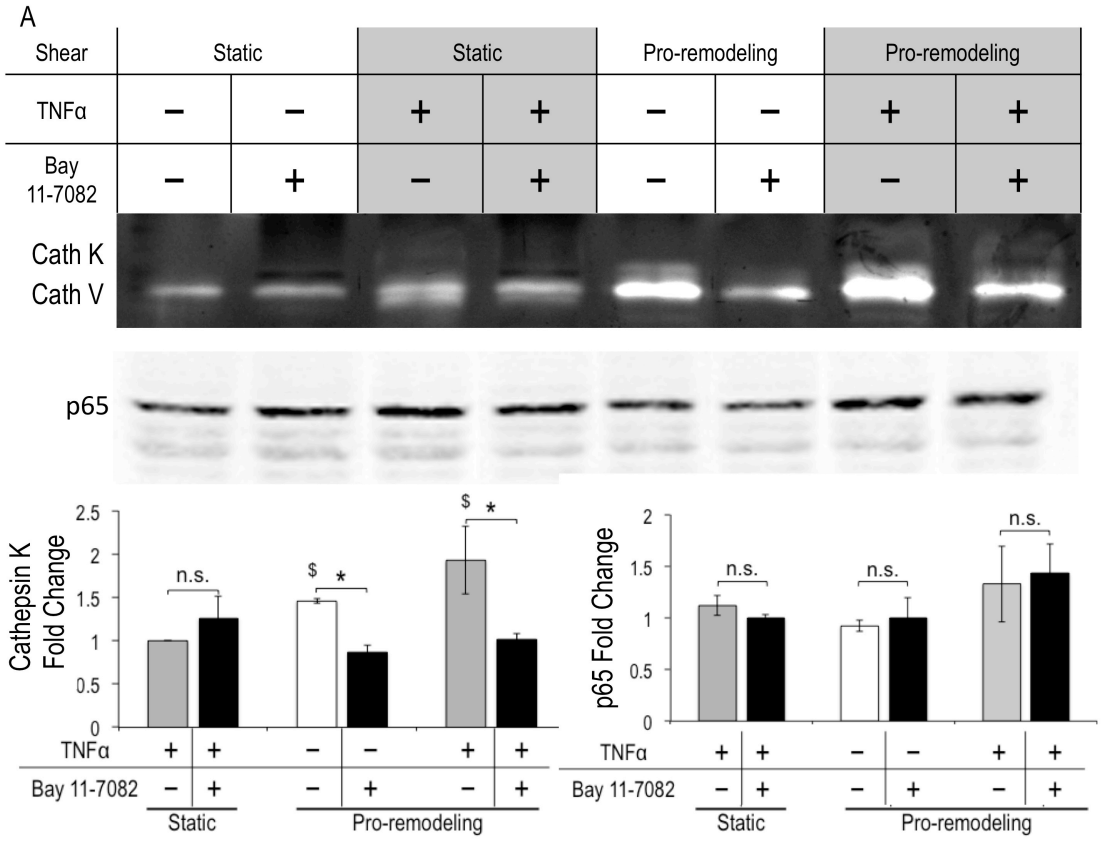
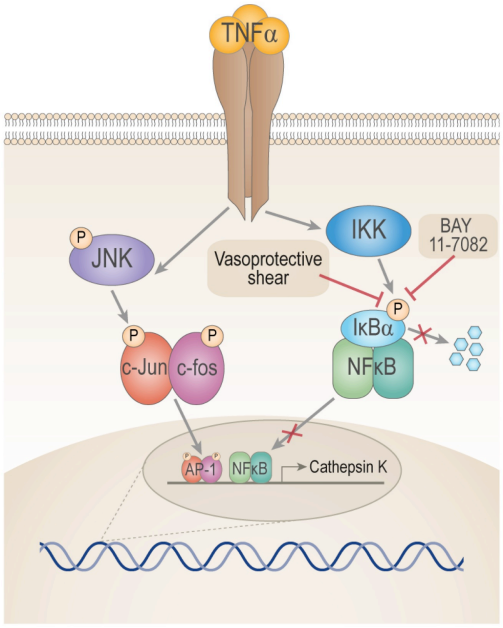


Figure 5-8: Shear stress-mediated activation of cathepsin K is dependent on NFκB activation. Confluent HAECs were pre-conditioned with 5μM of Bay 11-7082, an inhibitor of NFκB activation, for 1 hour prior to being stimulated with or without 10ng/mL TNFα, and maintained in static (lanes 1, 2, 5, 6), or pro-remodeling (lanes 3, 4, 7, 8) shear stress for 20 hours. Cultures were lysed and (A) cathepsin activity was determined using multiplex cathepsin zymography, and NFκB protein levels were determined using Western blot, and (B) quantified using densitometry analysis. Pro-remodeling shear stress alone induced detectable levels of cathepsin K activity, but inhibition IκBα phosphorylation prevented detectable cathepsin K activity.



TNFα		CatK activity	JNK	NFκB
Vaso-protective	−	−	−	−
	+	−	+	−
Pro-remodeling	−	+	−	+
	+	++	+	+

Figure 5-9: Schematic representation of biomechanical and biochemical regulation of cathepsin K activity in endothelial cells. Proposed mechanism by which vasoprotective shear stress inhibits cathepsin K protein expression and activation downstream of TNFα stimulation via reduced NFκB activation and expression.

maintained under vasoprotective shear stress suggests that transcriptional activation cathepsin K gene may be dependent on the ability for both c-Jun and NFκB to bind to the promoter region.

Vascular biomechanical studies have established that regions of the vasculature exposed to vasoprotective shear stress are protected from vascular remodeling, while regions exposed to pro-remodeling shear stress are more vulnerable [59, 80, 88, 152, 49, 32]. Work done by our group has previously correlated increased active cathepsin K levels at sites of disturbed flow to arterial remodeling [111, 110]. Additionally, the initiation of arterial remodeling pathologies is the result of endothelial cell dysfunction

and activation from inflammatory cytokines, such as TNFα [151, 71]. The findings from this study provide a mechanistic link for how arterial endothelial cells integrate biomechanical shear stress and inflammation to differentially regulate cathepsin K, which has particular relevance for those suffering from sickle cell disease. People with sickle cell disease suffer from a chronic inflammatory syndrome characterized by elevated TNFα and chronic activation of vascular endothelial cells, which may induce upregulation of cathepsin K throughout the vasculature. Additionally, transient aggregation of red and white blood cells along the arterial wall may generate spontaneous regions of pro-remodeling shear stress [11], allowing regions of the vasculature to become vulnerable to cathepsin-mediated arterial remodeling. The findings from this study implicate that c-Jun phosphorylation and NFκB are necessary for up-regulation of cathepsin K. Work done by others have found NFκB activity to be increased in regions of pro-remodeling shear stress in mice, and *in vivo* inhibition of NFκB protects against atherosclerotic lesion formation [43, 51]. Furthermore, TNFα stimulation of HAECs stimulates NFκB to complex with phosphorylated c-Jun and bind to the TPA response element in human aortic endothelial cells [117, 126], which is present in the cathepsin K promoter region. Therefore, we conclude that TNFα is sufficient to induce cathepsin K activity only if there is sufficient NFκB availability to allow for activation of the TPA response element on the cathepsin K promoter region; loss of NFκB signaling or expression, as is seen after exposure to vasoprotective shear stress or through inhibition with Bay 11-7082, significantly cathepsin K expression and activity.

The identification of NFκB as a shear-responsive activator of cathepsin K expression provides a novel therapeutic target for for people suffering from sickle cell disease, especially children who have a 220-fold increase in the chance of stroke before the age of 20 as a resulting from advanced arterial remodeling of the middle cerebral and carotid arteries [133, 3, 4, 2, 116]. Based on previous work establishing

cathepsin-mediated arterial remodeling and elastin degradation in other vascular diseases, such as atherosclerosis, in conjunction with our findings that TNFα increases active cathepsin K activity in large artery endothelial cells [68], we hypothesize that pathological activation of cathepsin K may also contribute to rapid cerebral arterial remodeling leading to stroke [67]. Due to the effects of elevated plasma levels of TNFα and it potential to induce cathepsin K expression and activity, combined with the altered rheology of sickle red blood cells and the possibility of spontaneous generation of pro-remodeling shear stress anywhere along the arterial tree, we posit that people with sickle cell disease are uniquely vulnerable to sustained, upregulation of cathepsin K within the arterial wall leading to rapid arterial remodeling. Although regular blood transfusions have been shown to mitigate the risk of stroke in young children with sickle cell disease, the chronic transfusions needed carry significant and life-threatening risks of alloimmunity, iron over load, and infection; however, this remains the only available therapy as there are currently no pharmaceutical treatment options available. By identifying other mechanistic pathways by which cathepsin activity is regulated by the hemodynamic and inflammatory environment of sickle cell disease, these may present new therapeutic targets for reduction in the risk of death for young children with sickle cell disease.

CHAPTER VI

SYSTEMIC INHIBITION OF JNK PREVENTS CATHEPSIN K MEDIATED VASCULOPATHY IN CAROTID ARTERIES OF SICKLE TRANSGENIC MICE

6.1 Introduction

Sickle cell disease is a genetic disorder affecting nearly 100,000 Americans and millions worldwide. Although commonly regarded as a hematological disorder characterized by the unique, "sickle" red blood cells, people with sickle cell disease have an 11% chance of a major stroke before the age of 20, and a 35% chance of a silent stroke before the age of 16; children are at the highest risk between 2 and 5 years old [10, 2, 115]. Post-mortem examination of the cerebral vasculature of children who have suffered strokes revealed significant luminal narrowing, as well as delamination and fragmentation of the internal elastic lamina of the internal carotid and middle cerebral arteries [133, 53, 116]. Moreover, in contrast to other cardiovascular diseases in which vascular remodeling develops over decades prior to clinical presentation, sickle cell disease induces arterial remodeling at a dramatically accelerated rate. Despite the high risk and rapid formation of arterial remodeling in sickle cell disease, there is little consensus on how these vascular lesions are initiated or progress. However, the rapid and extensive remodeling of the carotid and cerebral arteries of young children with sickle cell disease led our group to hypothesize that pathological proteolytic remodeling may play an important role in vascular lesion development.

Research on the development of pathological arterial remodeling have indicated that specific, predictable regions of the vascular tree are vulnerable, specifically sections that are characterized by sharp bends or bifurcations, such as the carotid and

cerebral arteries, due to the generation of low, oscillatory shear stress [152, 73, 49, 32]. Additionally, work done by our group and others has elucidated that one of the key effects of sustained oscillatory blood flow is the activation and secretion of powerful proteases into the arterial wall, leading to degradation of the elastic lamina, a principle initiating step of further pathological remodeling. As mentioned previously, we are interested cathepsin K's involvement in arterial remodeling in sickle cell disease due to it being a potent elastase and its established role in other cardiovascular diseases. In addition to regions of sickle-mediated arterial remodeling, namely the carotid and cerebral arteries, being identified as inherently vulnerable to increased cathepsin activity, our work has identified that the often overlooked chronic inflammatory syndrome generated by sickle cell disease also generates a perpetual pro-cathepsin environment within the vasculature. People with sickle cell disease have chronically elevated inflammatory cytokines, such as tumor necrosis alpha (TNFα), which is sufficient to induce cathepsin K in arterial endothelial cells via JNK and c-Jun signaling [68]. Additionally, people with sickle cell disease have higher numbers of circulating monocytes in the peripheral blood, and these monocytes are also uniquely conditioned to also be able to induce cathepsin K activity in endothelial cells, again dependent on JNK and c-Jun signaling [67]. Therefore, it is apparent that the chronic inflammation characteristic to sickle cell disease, in conjunction with the natural geometry and hemodynamics of the carotid and cerebral arteries generate a powerful pro-cathepsin environment for the development and progression of arterial remodeling.

Developed in the late 1990s, the Townes transgenic murine model successfully recapitulates many of the symptoms and comorbidities seen in humans [16]. Mice were genetically modified such that the murine hemoglobin genes were knocked out and human hemoglobin genes, including those that contain the sickle mutation, were knocked in. However, there remains a need for more comprehensive characterization of the vascular and neural pathologies associated with sickle cell disease in the Townes

model. Furthermore, a more complete characterization of the animal model would be benefit the continued investigation and development of pharmacological therapeutics for the prevention and treatment of stroke in sickle cell disease. Therefore, the purpose of this study was three-fold. First, we tested the hypothesis that sickle transgenic mice develop vasculopathies analogous to what is observed clinically in the cerebral and carotid arteries leading to stroke in the Townes model of sickle cell disease. Second, we tested the hypothesis that vascular remodeling *in vivo* was the result of pathologically elevated cathepsin K activity within the arterial wall. Third, we hypothesized that inhibition of JNK signaling in vivo, based on our previous findings in vitro, would be sufficient to reduce cathepsin K expression and activity, thereby preventing the initiation and progression of arterial remodeling in sickle cell disease.

6.2 Methods

6.2.1 Animals

Townes sickle transgenic mice (B6; 129-Hbatm1(HBA) Tow Hbbtm2 (HBG1,HBB*) Tow/Hbbtm3 (HBG1,HBB) Tow/J) were obtained from Jackson Laboratories and used to establish a breeding colony in the Physiology Research Laboratory at Georgia Tech. Animals were housed according to a 12:12 light:dark cycle and fed an ad lib diet. Pups born to breeders were sexed and separated into different cages at 21 days old. All mice were housed and all experiments were conducted with approval of the Georgia Institute of Technology Institutional Animal Care and Use Committee (protocol number A13011) and the supervision of the Physiology Research Laboratory veterinarian technologists.

6.2.2 Identification of Normal and Sickle Hemoglobin

Animals were evaluated for entry into the drug trial based on sickle status at 21 days old. Animals were anesthetized using an 3% isoflurane and approximately 600μL of whole blood was obtained via retro orbital blood draw into heparinized blood

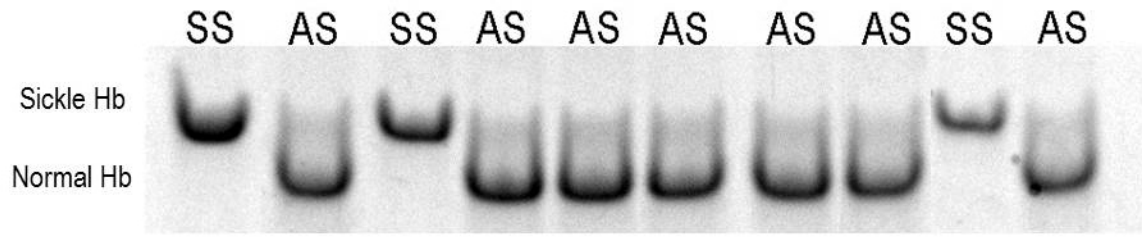


Figure 6-1: Identification of sickle status of transgenic mice using Native PAGE. Whole blood samples were obtained via retro-orbital blood collection and lysed with 1mL of distilled water. Hemoglobin was run using standard Native PAGE to isolate normal hemoglobin at a smaller hydrodynamic radius, resulting in faster migration and development of a lower band. Sickle hemoglobin has a larger hemodynamic radius, resulting in slower migration and development of a higher band. Representative image is shown.

collection tubes. Whole blood was lysed with 500μL 100% dH2O and spun at 90,000xg for 10 minutes to pellet cellular debris leaving hemoglobin within the supernatant. The hemoglobin was then categorized based on the electrophoretic migration distance through a standard Native PAGE protocol. Briefly, the supernatant was diluted 1:8 in a 50% glycerol solution and 20μL was loaded into a modified 12.5% polyacrylamide gel lacking SDS. The gel was run for 2 hours at 100V using a 3% Tris base and 14.18% glycine running buffer. Gels were imaged using an ImageQuant 4010 system (GE Healthcare). Since hemoglobin containing the sickle mutation has a slightly more positive electrical charge, compared to normal hemoglobin, sickle hemoglobin will not migrate as far through a Native PAGE gel. Therefore, animals with a single, high hemoglobin band were classified as homozygous for sickle cell disease (SS), animals with a doublet band were classified as sickle trait (AS), and animals with a single, low hemoglobin band were classified as normal (AA, Fig 6-1).

6.2.3 JNK Inhibition

For the JNK inhibition drug trial, 9 animals were selected at 21 days old and sickle status was determined; a total of 2 AA animals, 4 AS animals, and 2 SS animals

were enrolled. Animal weights were determined at the onset and weekly measurements were taken for the duration of the drug study. The inhibitor of JNK activity, SP600125 (Invitrogen), was diluted in 10% DMSO and injected intraperitoneally at a concentration of 50mg/kg of animal body weight. Injections were performed daily over the course of 8 weeks, and animal weights were monitored according to IACUC policy as an indicator of general animal health.

6.2.4 Tissue Isolation

Animals were euthanized after 8 weeks using CO2 asphyxiation. After termination, the animal's thoracic cavity was exposed and a 28 gauge catheter was inserted 2-4mm through the apex of the heart and into the left ventricle. The ascending vena cava was cut and the circulatory system of the animal was perfused with via gravity-driven system with physiological saline for 5 minutes to remove residual blood. The spleen, along with the thoracic aorta, heart, left and right common carotid arteries, and brain was then isolated. Relative size and weight of the heart and spleen were measured and recorded. The thoracic aorta was cleaned of surrounding adventitia, and placed in 60μL of zymography lysis bufferLi:2010km, Wilder:2011gk with 0.1mM leupeptin. Additionally, the carotid arteries were carefully cleaned of adventitia and placed in 10% neutral buffered formalin for 30 minutes, and then embedded in HistoGel (Richard-Allen Scientific). Brains were removed via craniotomy and placed in 10% neutral buffered formalin for 24 hours.

6.2.5 MRI scans of sickle transgenic mice brains

After fixation, brains were immersed and embedded in a 2% agarose (Phenix Research Products) with 2mM Gadolinium(III) oxide (Acros Organics) overnight. The brains were then scanned using a Bruker 9.4 Tesla magnet with a T2-weighted protocol (TE = 13.4 ms. TR= 10,000, 512 x 512 x 70).

6.2.6 Arterial Elastin Morphology

HistoGel-embedded carotid arteries and isolated brains, not selected for MRI imaging, were imbedded were paraffin processed and 5μm sections were obtained and select serial sections were deparaffinized. Elastin was visualized using a modified Verhoeff elastic-van Gieson stain (Electron Microscopy Sciences). Briefly, sections were placed in a modified Verhoeff elastic solution (3 parts 3% Hematoxylin, 2 parts 2% ferric chloride, 1 part Lugol's idodine) for 7 minutes, and washed with warm water for 1 minute. Samples were then resolved in 0.4% ferric chloride for 75 seconds, rinsed again with warm water for 5 minutes, and counter-stained with Van Gieson's solution for 60 seconds. Slides were then dehydrated, cover-slipped, and imaged with a Ti-Eclipse microscope (Nikon Instruments). For elastin fragmentation quantification, carotid artery sections were blinded and imaged under 40x magnification, and areas of elastin fraying, fragmentation, and delamination were identified and recorded by eye (n3, p¡0.05).

6.2.7 Immunohistochemistry for Cathepsin K

Deparaffinized sections of carotid arteries or brain tissue were permeabilized with 0.2% Triton X for 15 minutes, washed 3x with PBS, followed by antigen retrieval using 0.1% trypsin for 15 minutes at room temperature. Samples were washed again 3x with PBS and blocked with 2% BSA for 1 hour at room temperature. Slides were stained with a 1:50 dilution of rabbit monoclonal anti-mouse cathepsin K (Santa Cruz Biotechnology) for 24 hours at 4C, washed in PBS, and stained again with AlexaFluor^{TM} 488 secondary antibody (Invitrogen). Slides were counter stained with a 1:20 dilution of AlexaFluor^{TM} 562 phallotoxin (Invitrogen) for 10 minutes to visualize actin filaments. Slides were washed a final time in PBS and mounted with Prolong Gold anti-fade mounting media containing DAPI (Invitrogen) for visualization of the nucleus.

6.2.8 Fluoro Jade B Staining of Degenerating Neurons

Select animals were fixed through perfusion of 10% neutral buffered formalin through the left ventricle of the heart using a gravity-fed perfusion system; brains were isolated and processed for paraffin embedding, 5μm sections were obtained and samples were dried for 30 min at 50C. Sections were postfixed with 4% neutral buffered formalin again, and washed 3x with PBS. Slides were incubated in 0.06% potassium permanganate for 10 minutes on a table shaker, and washed twice with distilled water, followed by incubation in 0.0004% Fluoro Jade B (Milipore) solution containing 0.1% acetic and 0.0004% DAPI solution for 20 minutes at room temperature. Slides were rinsed 3x for 2 minutes, and placed in an over at 50C until fully dried. Finally, slides were cleared by immersion in xylene for 2 min before cover slipping with Prolong Gold mounting medium (Invitrogen).

6.2.9 Multiplex Cathepsin Zymography

Multiplex cathepsin zymography was used to assess cathepsin activity in the arterial walls of mouse thoracic aortas. Excised suprarenal were placed in 50μL of lysis buffer (20 nM Tris-HCl [pH 7.5], 5mM ethylene glycol tetraacetic acid [EGTA], 150mM NaCl, 20mM β-glycerol phosphate, 10mM NaF, 1mM sodium orthovanadate, 1% Triton X-100, and 0.1% Tween 20) with 0.1mM leupeptin, homogenized using disposable sample grinders (GE Healthcare). Additionally, whole brain lysates were obtained through use of a neural tissue dissociation kit (Miltenyi Biotec). Briefly, whole brains were minced in ice cold PBS and enzymatically dissociated at 37 degrees C for 15-20 minutes. The solution was then passed through a 40 μm cell strainer to render a single cell suspension. Cells were pelleted at 500xg for 10 minutes, and lysed with 60μL of lysis buffer with 0.1mM leupeptin added fresh. Protein concentrations were obtained by the bicinchoninic acid (BCA) assay (Pierce). Cathepsin zymography was performed as described previouslyLi:2010km, Wilder:2011gk, Hansen:2012ef,

Hansen:2013gc. Gels were imaged using an ImageQuant LAS 4000 (GE Healthcare).

6.2.10 Statistical Analysis

Each experimental condition was repeated with a minimum of three biological replicates, and each data point is presented as the mean value and standard error of the mean. Representative images are shown.

6.3 Results

6.3.1 Sickle Cell Disease Promotes Cathepsin Mediated Elastin Degradation of the Carotid Arteries

After identification of each animal as normal, trait, or sickle transgenic, animals were inspected for hallmark pathologies associated with sickle transgenic animals. Spleens from euthanized animals were excised and measured. Normal (AA) and trait (AS) spleens were of comparable size, but sickle spleens were substantially larger, consistent with both human pathophysiology (Fig 6-2, representative image shown). Additionally, hearts from each animal were also isolated and measured for relative size differences. Again, there was no substantive difference between normal and sickle trait animals, but sickle transgenic animals had larger hearts, specifically with regards to the left ventricle Fig 6-3, suggesting that sickle transgenic mice have increased peripheral resistance caused by vasooclusion.

We first investigated if sickle cell disease in transgenic animals induced vascular remodeling similar to what has been clinically observed in humans. 5μm sections of the common carotid arteries isolated animals after gravity perfusion fixation with 10% neutral buffered formalin (NBF), prepared for paraffin histology. Elastin morphology was visualized through modified Verhoeff elastic-van Gieson stain. Sickle transgenic (SS) mice had 8-times more elastin fragmentation compared to sickle trait (AS) animals (Fig 6-4, orange arrowheads); however, there was no significant difference between AS animals and normal (AA) animals (data not shown).

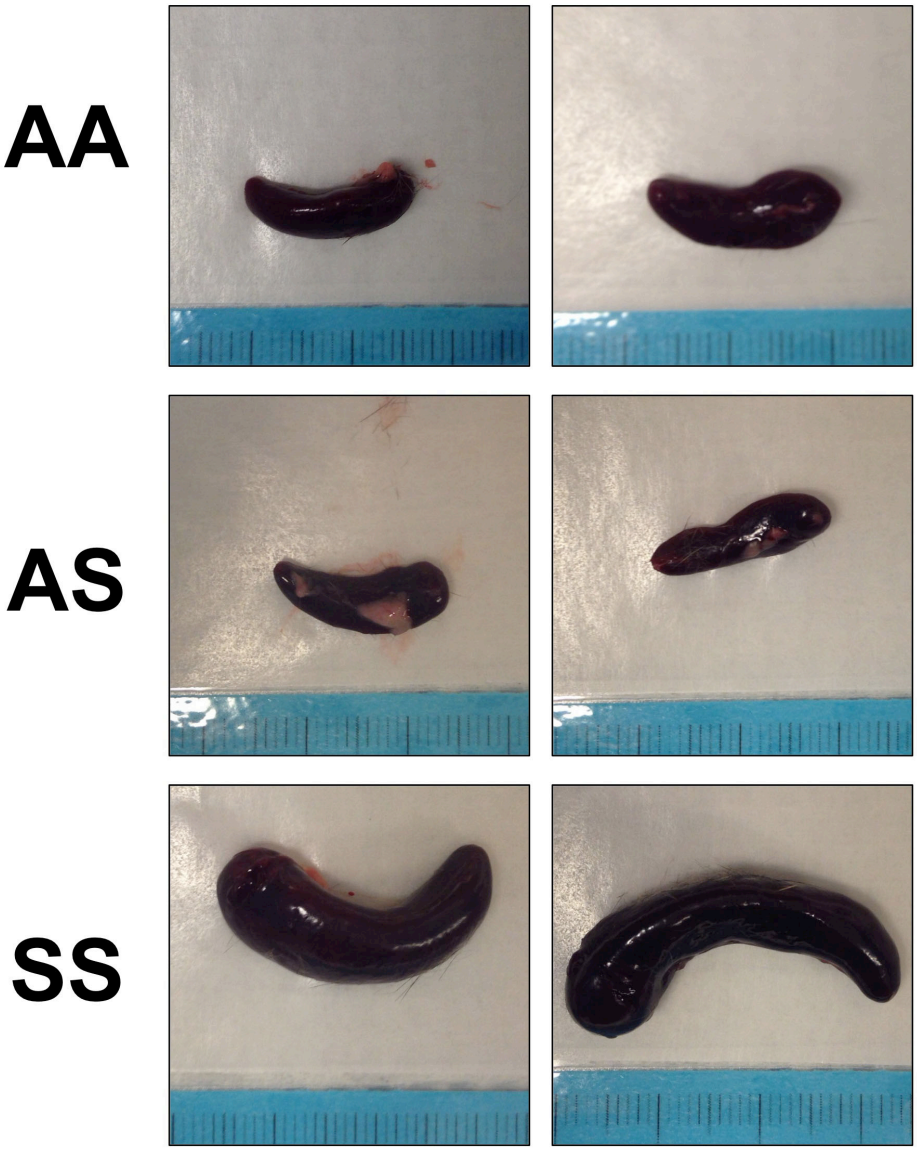


Figure 6-2: Comparison of spleen sizes isolated from normal and trasngenic sickle mice. Normal (AA), trait (AS), and sickle transgenic (SS) mice were euthanized and the spleens were isolated and measured along their longest axis. Spleens from normal and sickle mice were of comparable length, and spleens from sickle animals were substantially larger; representative images shown.

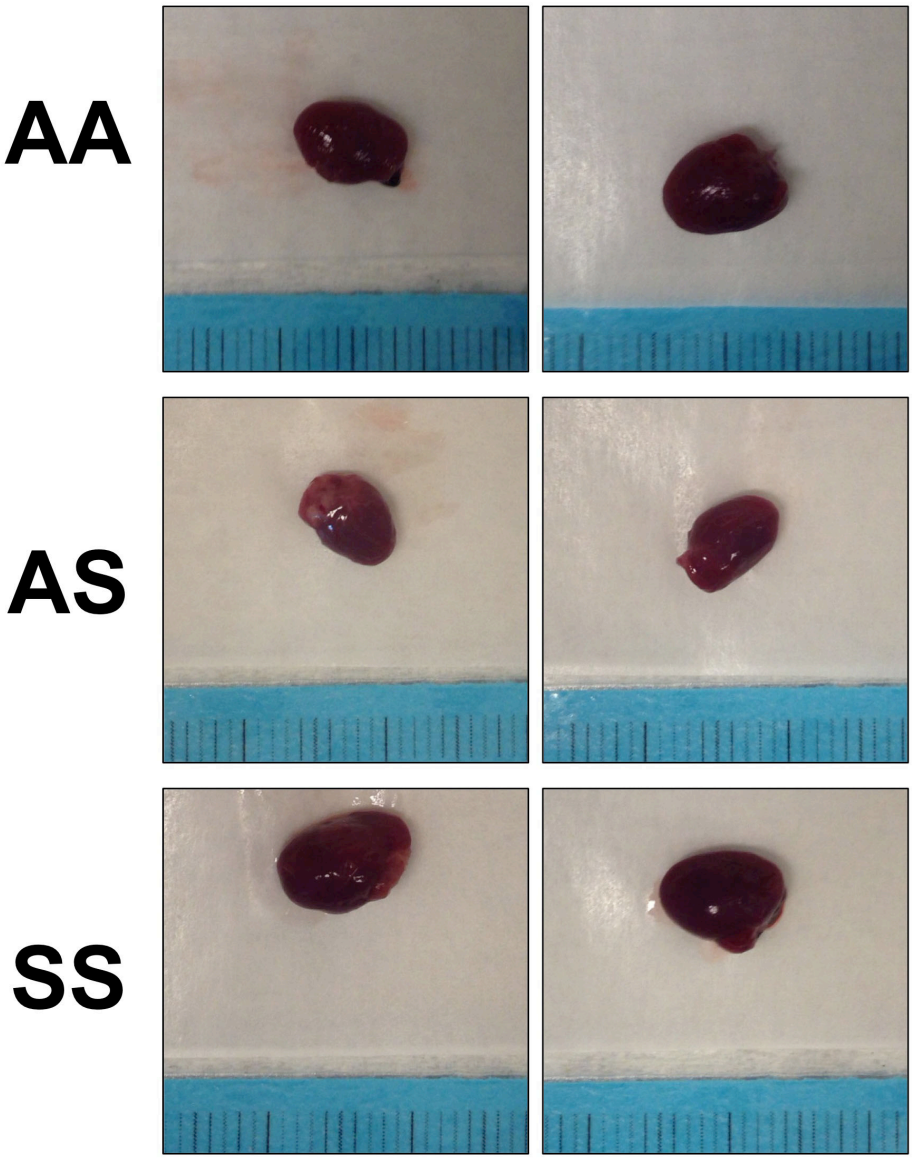


Figure 6-3: Sickle cell disease increases size of left ventricle in transgenic mice. Hearts were isolated from normal (AA), trait (AS), and sickle transgenic (SS) mice and measured to determine relative size differences. Normal and trait animals had comparable heart sizes, but sickle transgenic animals were markedly larger.

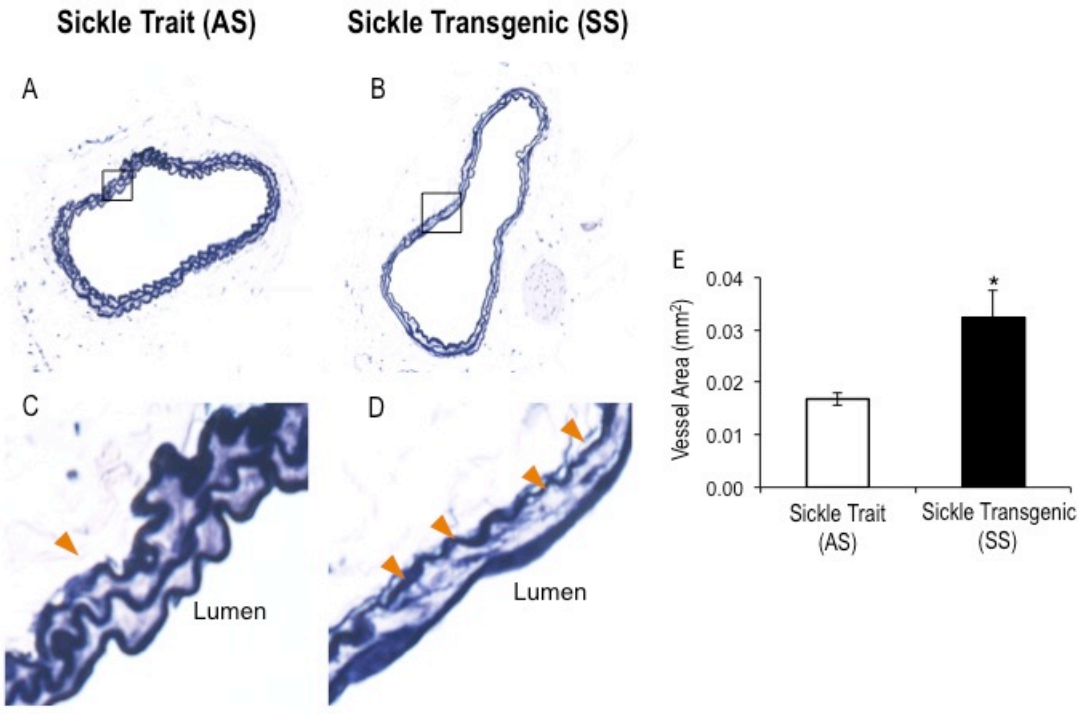


Figure 6-4: Sickle cell disease promotes elastin fragmentation in transgenic mice. Perfusion fixed carotid arteries from trait and transgenic mice were isolated, sectioned, and stained for elastin morphology using modified Verhoeff elastic-van Gieson stain. (A-C) Sickle transgenic mice had significantly smaller luminal areas, and (D) significantly increased incidence of elastin fragmentation, compared to trait mice. * denotes $p<0.05$, $n=4$.

Furthermore, carotid arteries in sickle transgenic mice had more linear elastin fibers compared to trait animals, indicated by a higher form factor, which suggests carotid arteries in these animals may have significantly altered biomechanical properties. Loss of elastin integrity also resulted in a significant increase in luminal area in SS mice, compared to AS mice, further implicating onset of pathological arterial remodeling (Fig 6-5; $p<0.05$, n=4.)

Work done previously by our group, and others, has implicated cathepsin K in cardiovascular remodeling pathologies, such as atherosclerosis. Furthermore, we have also shown that the circulatory environment in sickle cell disease is sufficient to induce elevated cathepsin K activity in arterial endothelial cells [67]. Therefore, we next investigated if areas of elastin fragmentation coincided with elevated cathepsin K expression. Serial sections of AA, AS, and SS carotid arteries were obtained and immunostained for cathepsin K protein levels. SS mice had substantially higher cathepsin K protein expression within the arterial, compared to both AA and AS animals (Fig 6-6). Furthermore, the elevated cathepsin K activity co-localized to regions of elastin fragmentation. These data suggest that sickle cell disease promotes elastin degradation and arterial remodeling through up-regulation of cathepsin K protein expression within the arterial wall. Next, we investigated the repercussion of sickle cell disease on the vessel integrity of the cerebral arteries.

6.3.2 Sickle transgenic mice develop cerebral vasculopathy and stroke

In addition to the carotid arteries, clinicians have identified that strokes associated with sickle cell disease occur as a result of remodeling and occlusion of the cerebral arteries. Sickle trait and sickle transgenic mice were euthanized and perfusion fixed using 4% PFA, followed by craniotomy to remove the brain. 5μm section were obtained and histologically stained with a modified Verhoeff elastic-van Gieson stain

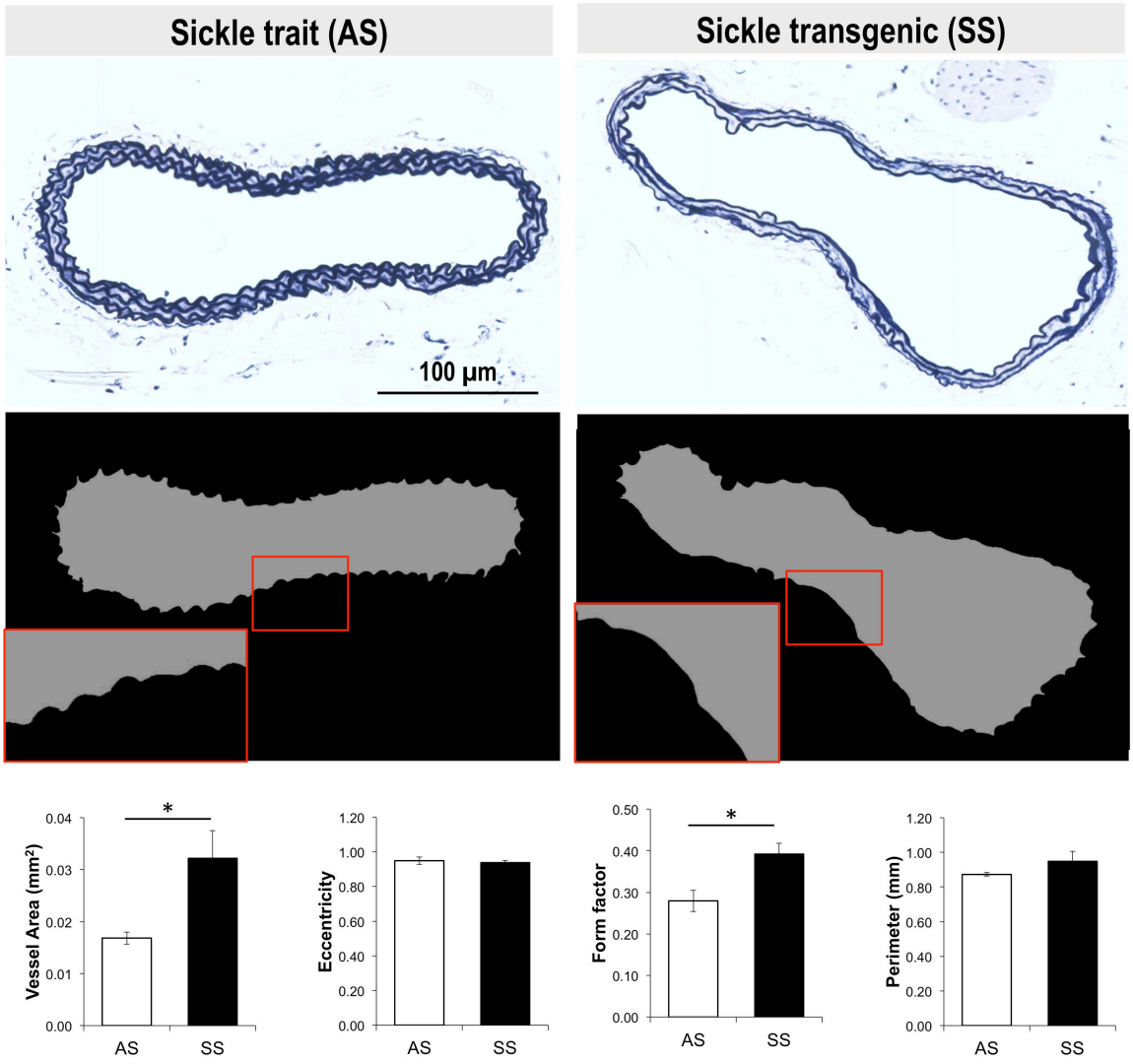


Figure 6-5: Sickle cell disease promotes arterial remodeling leading to increased luminal area of carotid arteries. Perfusion fixed carotid arteries from AS and SS mice were isolated, sectioned, and stained for elastin morphology using modified Verhoeff elastic-van Gieson stain. Images were analyzed using photomasking and image analysis to quantify luminal area, eccentricity, form factor, and perimeter. * denotes $p<0.05$, $n=4$.

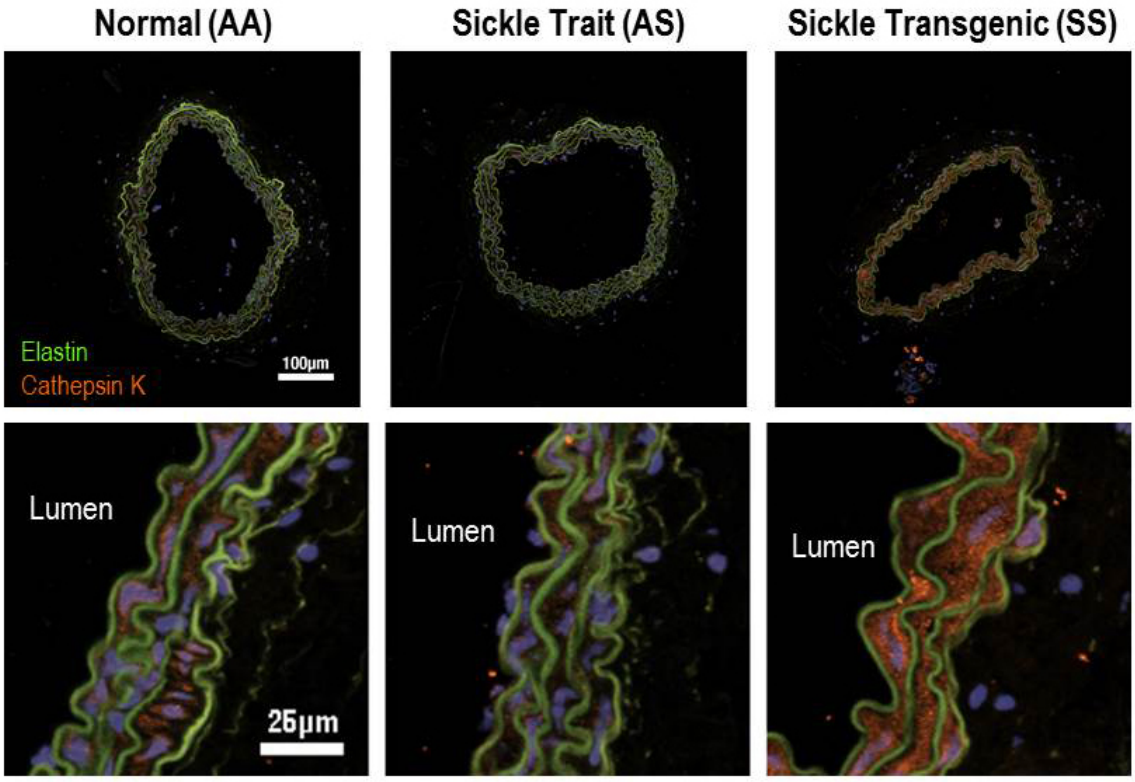


Figure 6-6: Sickle cell disease upregulates cathepsin K in transgenic mice. Perfusion fixed carotid arteries from trait and transgenic mice were isolated, sectioned, and immunostained for the presence of cathepsin K protein. Only sickle transgenic animals possessed positive cathepsin K staining (orange) in the arterial wall, with undetectable levels in normal and trait animals; elastin fibers are autofluorescent (green).

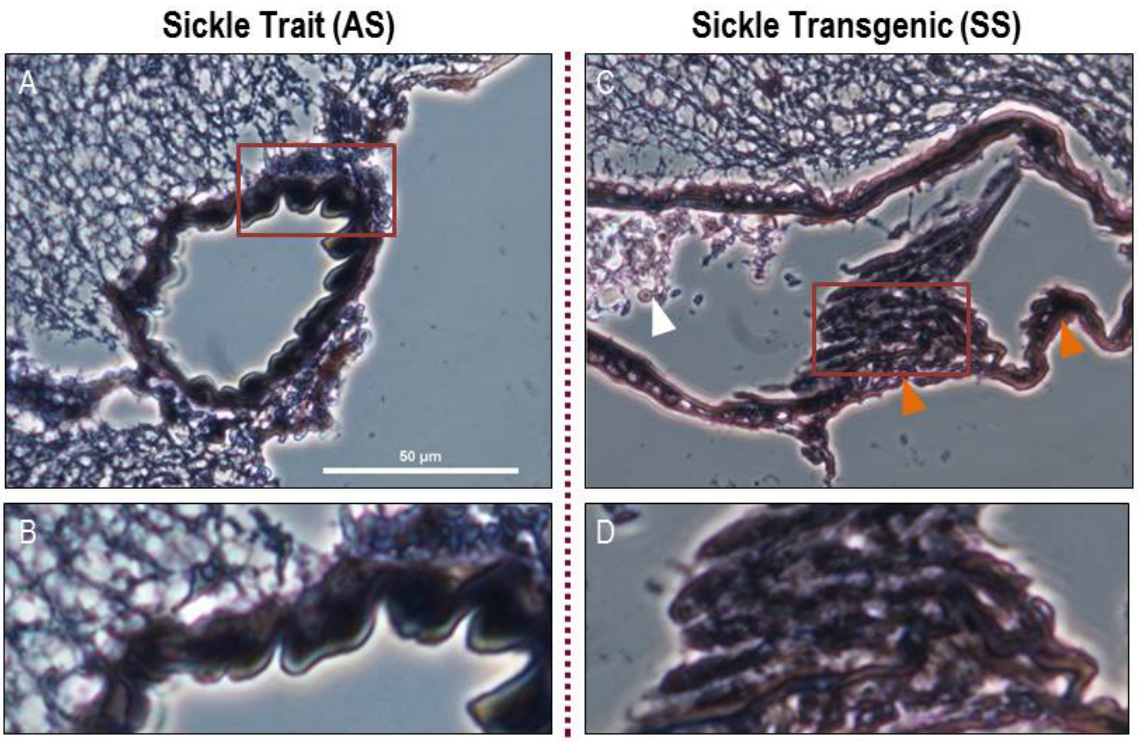


Figure 6-7: Sickle transgenic mice develop luminal narrowing and elastin remodeling in middle cerebral artery. Histological sections of whole brains were obtained and stained for elastin morphology using modified Verhoeff elastic-van Gieson stain, and the middle cerebral artery was imaged. Sickle transgenic animals present advanced elastin remodeling (orange arrowheads), as well as aggregations of blood cells along the vessel wall (white arrowheads); trait animals did not present any detectable elastin remodeling or cell aggregations.

to visualize elastin structures. Sickle transgenic animals presented regions of significant remodeling within the middle cerebral artery characterized by pathological elastic reorganization (orange arrowheads), luminal narrowing, and aggregations of red blood cells along the arterial wall (white arrowheads); AS animals had no discernible remodeling, elastin fragmentation, or accumulation of circulating red blood cells (Fig 6-7). These data suggest that sickle cell disease uniquely induces cerebral arterial remodeling, as well as promotes aggregations of circulating blood cells along the luminal wall, potentially leading to arterial dysfunction and obstruction of blood flow.

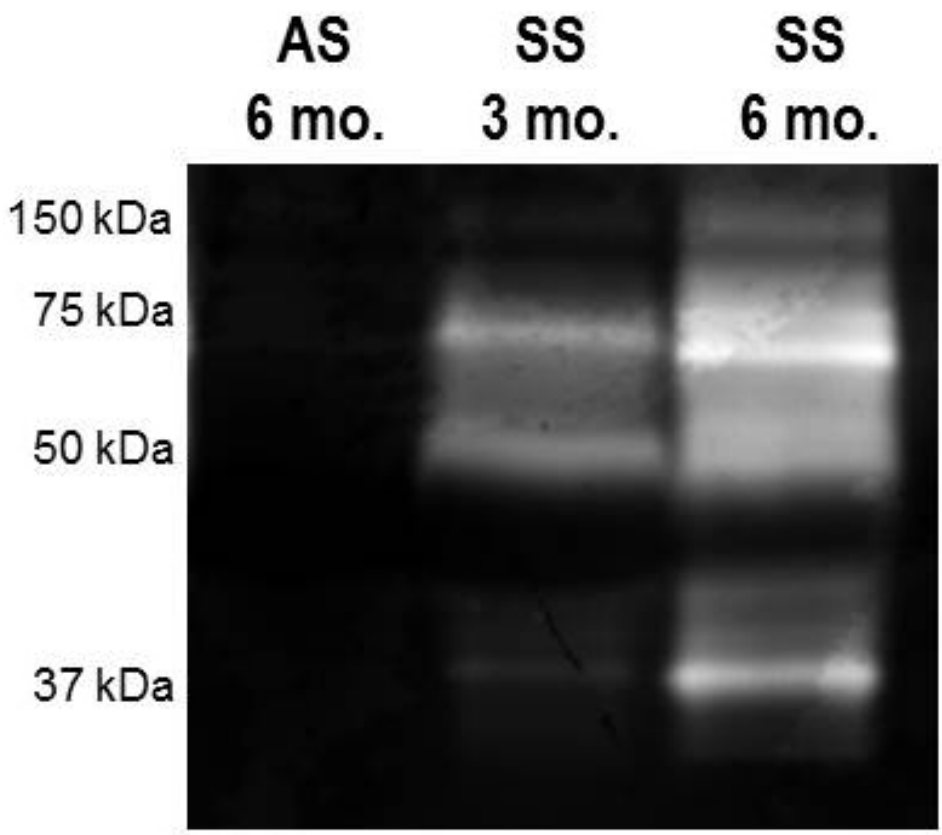


Figure 6-8: Brains from sickle transgenic mice have elevated cathepsin activity. Whole brains were isolated from 12-24 week old trait and transgenic mice, enzymatically dissociated, lysed, and cathepsin activity was assayed using multiplex cathepsin zymography. Brains from sickle transgenic mice have markedly increased cathepsin activity, compared to trait animals; cathepsin activity also appears to increase with age.

Additionally, brains from 24-week-old AS mice, as well as 12- and 24-week-old SS mice were isolated without perfusion fixation. Wholes brains were enzymatically disassociated, and the resulting single cell suspension was collected and lysed for zymography. Interestingly, SS mice had substantially higher levels of active cathepsins, which increased with age; ASS animals had no detectable cathepsin K activity (Fig 6-8). These results suggest that, like the carotid arteries, sickle cell disease also induces in vivo arterial remodeling of the cerebral arteries, potentially through pathologically elevated cathepsin K activity.

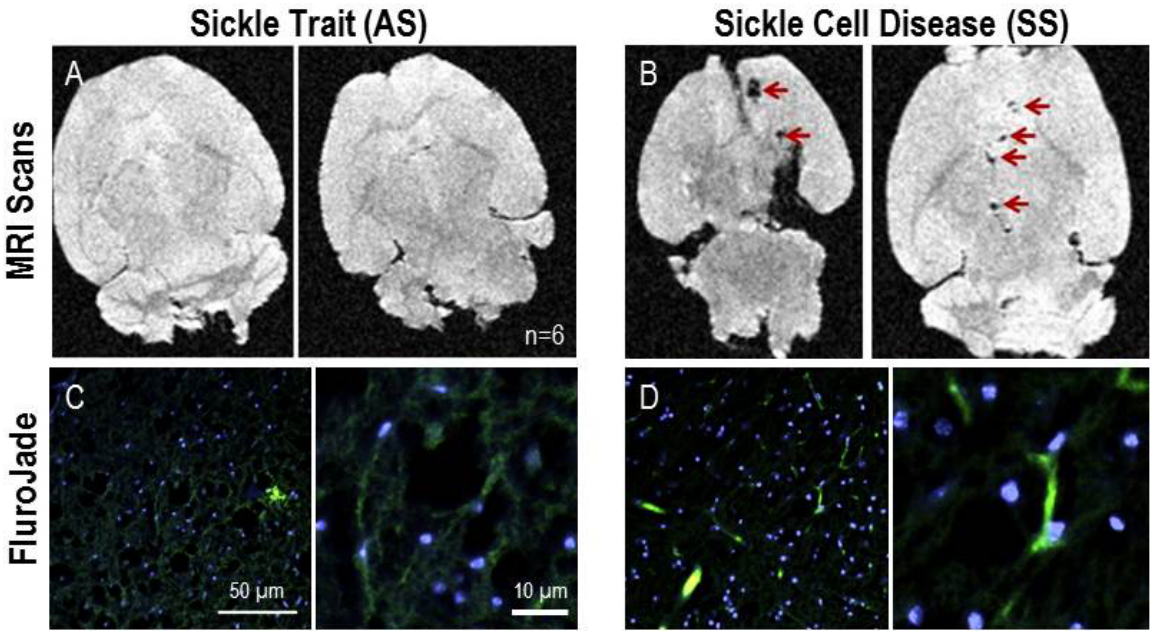


Figure 6-9: Sickle cell disease generates potential stroke lesions and increase neuronal death in transgenic animals. Isolated brains from sickle trait or transgenic animals were imbedded in gadolinium (III) oxide-agarose gel and imaged using a 9.4T animal MRI scanner running a T2-weighted modality. (A-B) Transgenic mice develop dark regions (red arrows) that are indicative of cerebral damage due to stroke. (C-D) Additionally, histological sections of whole mice brains were stained with FluroJadeB to identify perimortem neuronal cell death (bright green). Sickle transgenic animals had higher incidence of positive FluorJadeB staining, compared to trait animals.

Based on the our findings of arterial remodeling evidenced by increased luminal area, form factor, and incidence of elastin fragmentation in the carotid arteries of SS mice, we also tested the hypothesis that transgenic animals developed stroke lesions akin to what is observed in humans. AS and SS mice were euthanized and perfusion fixed, as previously discussed, and the brains were isolated and imbedded in a gadolinium (III) oxide-agarose gel. The brains were subsequently imaged using with a 9.4-Tesla MRI scanner running a T2-weighted imaging modality. Examination of the scans indicated both the sickle transgenic mice develop brain lesions indicative of stroke (Fig 6-9, red arrows), while sickle trait animals do not develop any lesions.

6.3.3 In vivo inhibition of JNK signaling reduces arterial cathepsin K expression and activity in sickle transgenic animals

Work done previously by our group has effectively established that the pro-inflammatory circulatory environment of sickle cell disease is sufficient to induce cathepsin K activity in large artery endothelial cells via JNK/c-Jun signaling [67, 68]. Additionally, the findings in this study have established a strong correlation between increased cathepsin K activity and expression in the carotid and cerebral arteries of sickle transgenic animals, exclusively. Therefore, we next determined if *in vivo* inhibition of JNK signaling would inhibit arterial cathepsin K and prevent elastin degradation, preserve arterial structure, and prevent stroke lesion formation. Nine mice were divided into drug treatment and vehicle groups based on genotype (Fig 6-10), and were given IP injections of 50mg/kg of the JNK inhibitor SP600125 daily for 8 weeks.

After, animals were euthanized and the thoracic aorta and common carotid arteries were isolated after perfusion fixation. Over the course of the 8 weeks, the systemic injections of SP600125 had no significant effect on the animals overall health, behavior, or weight. Tissue lysates were collected from the thoracic aortas and system inhibition of JNK activity was quantified by immunoblotting for c-Jun phosphorylation. The SS animal and AS animals had increased baseline levels of phosphorylated c-Jun, compared to the normal animals (Fig 6-11). Additionally, all mice injected with SP600125, regardless of sickle status, exhibited substantial reduction in c-Jun phosphorylation after 8 weeks of injection, compared to the vehicle control group (Fig 6-11) indicating that daily injections of the inhibitor SP600125 were sufficient to reduce physiological levels of JNK activity in arteries.

Having established that the JNK inhibitor successfully reduced JNK/c-Jun signaling within arteries of the mice, we next investigated if the effect of sustained JNK inhibition on the development of sickle cell disease related vasculopathy. $5/mu$m histological sections of the common carotid artery were obtained and serial sections were

Initiation of Trial			
Experimental Conition	Animal 1 (g)	Animal 2 (g)	Average Weight (g)
AA Ctrl	17	N/A	17
AS Ctrl	18	18.4	18.2
SS Ctrl	17	N/A	17
AA JNK	17	N/A	17
AS JNK	18.1	18.1	18.1
SS JNK	20.4	N/A	20.4

Completion of Trial			
Experimental Conition	Animal 1	Animal 2	Average Weight (g)
AA Ctrl	20.5	N/A	20.5
AS Ctrl	21.6	22.5	22.05
SS Ctrl	20.9	N/A	20.9
AA JNK	19.6	22.1	20.85
AS JNK	26.2	26.1	26.15
SS JNK	22.4	N/A	22.4

Figure 6-10: Sickle status, age, and bodyweight of animals receiving daily injections of SP600125. Littermate mice were blood typed to determine sickle status, and placed into the drug treatment or placebo groups. Animal ages and weights were recorded at initiation and termination of the study as indicators of general animals health. There was no substantial difference in animal weights in the drug versus placebo group.

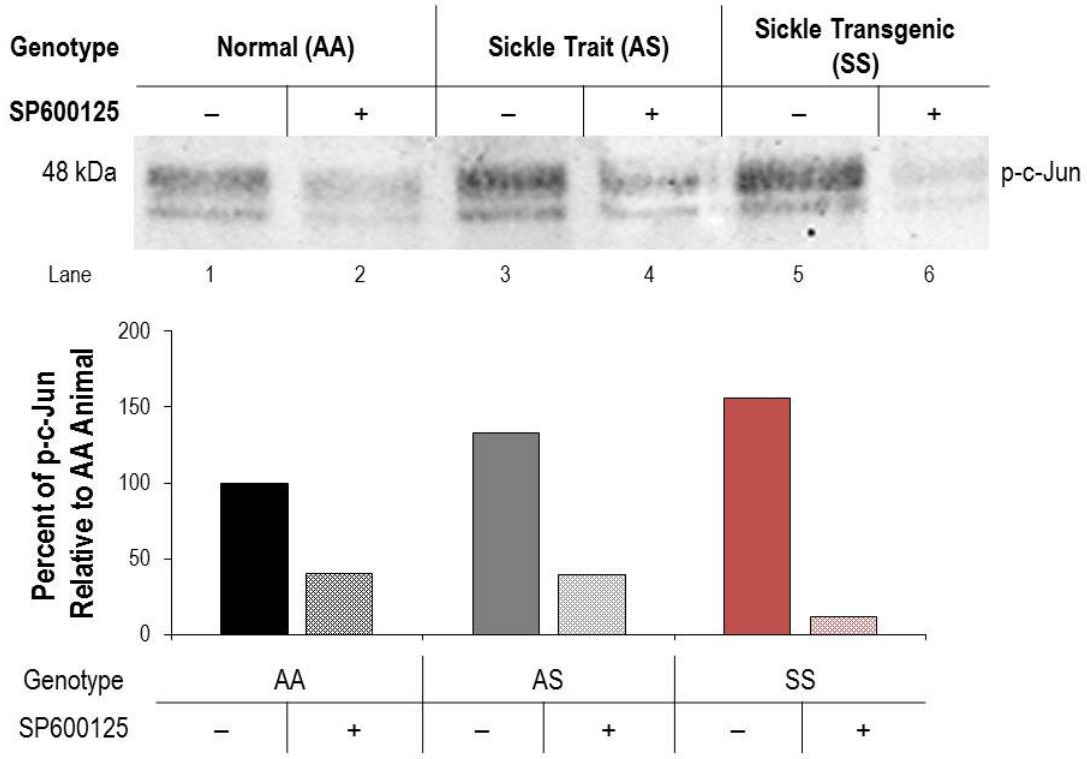


Figure 6-11: Intraperitoneal injections of SP600125 reduces arterial c-Jun phosphorylation. Thoracic aortas were isolated from drug and placebo group animals, and whole tissue lysates were assessed for c-Jun phosphorylation via Western blot. Sickle trait and transgenic animals had elevated phosphorylated c-Jun compared to normal animals, but all participants had marked decrease in c-Jun phosphorylation after injections of SP600125.

stained for elastin morphology and cathepsin K protein expression. As expected, the SS mice had 11 times more instances of elastin fragmentation within the wall of the carotid artery, compared to AA and AS mice (Fi 6-12). Furthermore, the total luminal area of carotid arteries from sickle mice were XX-times greater, compared to both normal and trait animals, indicating increased degradation of the elastin fibers within the arterial wall leading to decreased circumferential strain (Fig). Interestingly, however, inhibition of JNK signaling reduced both the instances of elastin fragmentation and luminal area of sickle transgenic animals to levels comparable to both treated and untreated AA and AS animals, suggesting that inhibition of the JNK/c-Jun signaling axis may be sufficient to prevent the development of sickle cell disease induced elastin degradation.

Based on our previous findings that cathepsin K activation is downstream of JNK/c-Jun signaling in vitro, and that cathepsin K protein co-localized with areas of elastin fragmentation in sickle transgenic mice, we next investigated the effect of in vivo inhibition of JNK signaling on cathepsin K protein expression in the carotid arteries of transgenic mice. Immunostianing for cathepsin K protein indicated a substantially higher level of protein expression throughout the arterial wall in SS animals, with little to no staining apparent in AS or AA animals. However, administration of the JNK inhibitor substantially reduced protein staining in the sickle transgenic animal, indicating that inhibition of JNK signaling inhibits sickle cell disease-induced cathepsin K expression in the arterial wall (Fig 6-13). Additionally, we assayed overall cathepsin activity in the mice using multiplex cathepsin zymography on tissue lysates obtained from the thoracic aortas. As expected, sickle transgenic mice had elevated total cathepsin activity, compared to normal and trait mice; however, inhibition of JNK signaling reduced cathepsin activity in transgenic, paralleling what was observed in the carotid arteries and previous in vitro studies (Figure 6-14). Taken together, we have established in this study that sickle cell disease results in over expression of

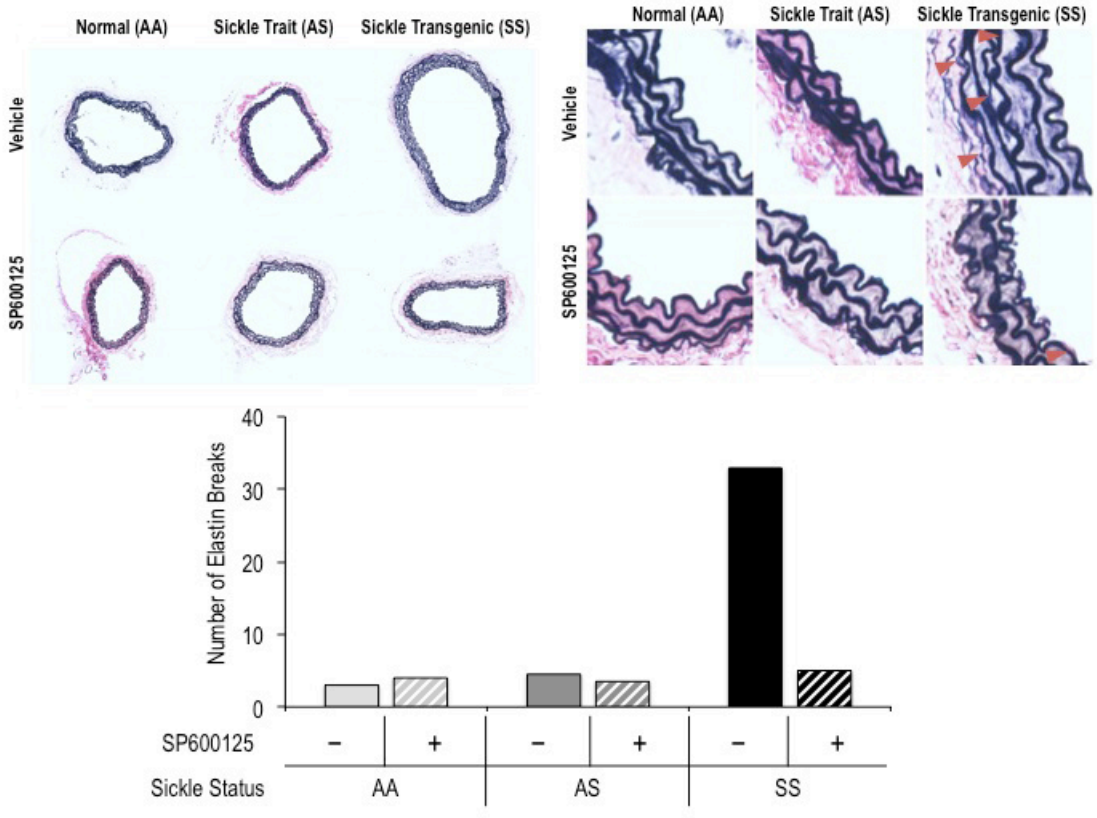


Figure 6-12: Inhibition of JNK signaling reduces elastin fragmentation in carotid arteries of transgenic mice. Carotid aortas were isolated from drug and placebo group animals, sectioned, and histologically stained for elastin morphology. Sickle transgenic animals receiving daily injections of the JNK-inhibitor SP600125 had substantially reduced incidence of elastin fragmentation compared to both trait and normal animals.

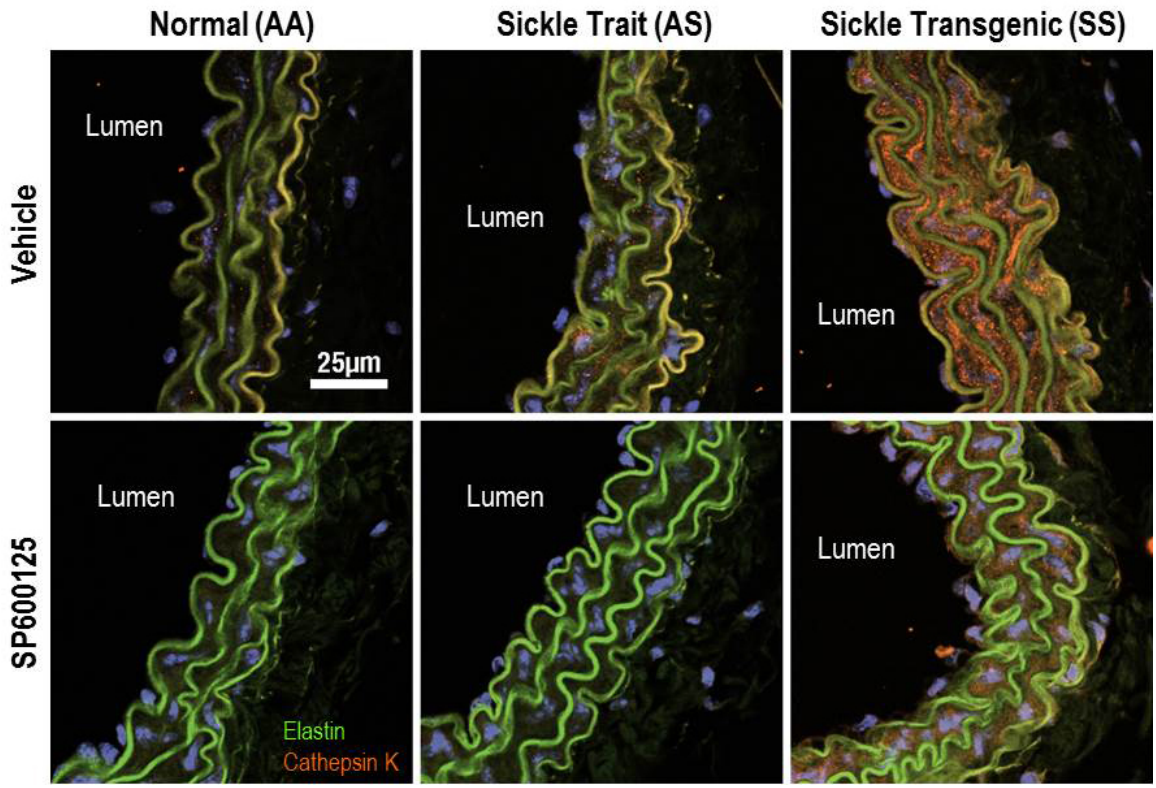


Figure 6-13: Inhibition of JNK signaling inhibits cathespin K protein expression in carotid arteries of transgenic mice. Carotid aortas were isolated from drug and placebo group animals, sectioned, and immunostained for cathepsin K protein. Sickle transgenic animals receiving daily injections of the JNK-inhibitor SP600125 had substantially weaker staining for cathepsin K, compared to the placebo group.

cathepsin K within the wall of the carotid artery, leading increased elastin fragmentation; however, systemic inhibition of JNK signaling is sufficient to reduce cathepsin K protein expression and activity, ultimately leading to better arterial integrity.

6.4 Discussion

In this study we are the first group to identify systematically characterize the development of vascular remodeling in the gold standard Townes sickle transgenic mouse model. Sickle transgenic mice present increased elastin fragmentation and remodeling in the carotid artery, evidenced by increased numbers of elastin breaks within

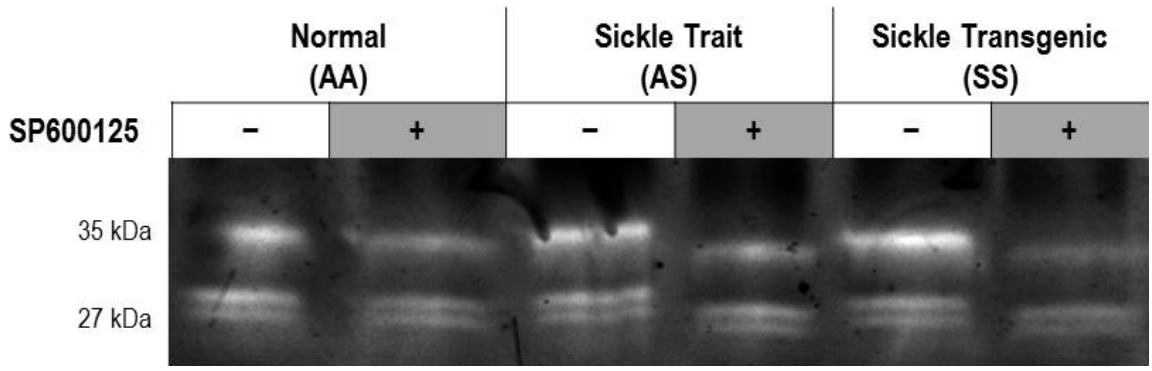


Figure 6-14: Inhibition of JNK signaling reduces arterial cathepsin activity in sickle transgenic mice. Whole tissue lysates were collected from the thoracic aortas of normal, trait, and transgenic mice and cathepsin activity was assayed using multiplex cathepsin zymography. Sickle transgenic animals had higher baseline cathepsin activity, but administration of the JNK inhibitor SP600125 substantially reduced activity.

the arterial wall (Fig 6-4). Furthermore, the luminal area of the carotid arteries of sickle mice was significantly larger than normal and trait mice, which is indicative of elastin degradation and loss of circumferential stress (Fig 6-5). Additionally, we determined that sickle transgenic mice have increased elastin fragmentation and pathological elastin restructuring within the middle cerebral artery (Fig 6-7), indicating that the Townes model of sickle cell disease recapitulates two important hallmarks of vascular degeneration found in human patients. Additionally, we not only identified that sickle cell disease induces substantially higher cathepsin K expression, that co-localizes with elastin degradation, in both the carotid and cerebral arteries, compared to both trait and normal mice, but that animals with sickle cell disease have higher cathepsin activity in their brain (Fig 6-7 and 6-6). Our group is also the first to identify increased neural death and the potential development of stroke lesions in sickle transgenic animals (Fig 6-9). In addition to being the first to describe and characterize the vascular pathology of sickle transgenic mice, our group was also able to use systemic inhibition of JNK signaling to reduce not only cathepsin K expression and activity in the carotid arteries of sickle mice, but also decrease the

elastin degradation observed in untreated animals.

Numerous investigations of sickle cell disease have focused on the generation of the vasoocclusive crises that forms due to aggregations of sickled red blood cells obstructing blood flow through the microvasculature, resulting in damage to bone [5], spleen [105], lungs [84], and many other organ systems, while simultaneously inducing extreme pain that can last anywhere from hours to days. In the past decade, research stemming from vasoocclusive crises has begun to examine the role of the abnormally adherent, stiff, oxidizing sickle red cells as an irritant that provokes an inflammatory response as it obstructs flow [114]. *In vivo* investigations using the Townes transgenic mouse model have implicated reperfusion injury as a possible source for the chronic inflammatory syndrome of sickle cell disease that is also observed in human subjects [63]. Ischemia followed by reoxygenation of the vasculature in sickle mice generated a distinct inflammatory response with increased rolling, adhesion, and emigration of vascular white blood cells facilitated by endothelial surface expression of adhesion molecules, implying that reperfusion following vasoocclusive crises in sickle cell disease results in activation of the endothelium, production of inflammatory cytokines like TNFα, promote circulation of abnormally high base-line leukocyte counts [146], as well as increased production of reactive oxygen species [153, 66], consistent with what is found in humans with sickle cell disease.

The presence of elevated inflammatory factors, activation of vascular endothelial cells, and increased monocyte adhesion and emigration into the vascular wall characteristic of vascular injury and inflammation in sickle cell disease are also known to be critical initiating events in the development of several vascular remodeling pathologies, such as atherosclerosis [151, 89, 37, 17]. During the progression of vascular remodeling diseases systemic inflammation and monocyte infiltration into the sub-endothelial space stimulate production and secretion of cathepsins, specifically cathepsin K. Previous work by our group has shown that both TNFα and circulating

monocytes isolated from people with sickle cell disease are sufficient to induce cathepsin K activity in vascular endothelial cells [68, 67], suggesting that the elevated TNFα and increased circulating monocytes reported by others may result in the substantially elevated levels of arterial cathepsin K reported in this study (Fig 3, 11). In addition to increased cathepsin activity, matrix metalloproteinase (MMPs), specifically MMP-2 and MMP-9, have also been implicated as mediators of vascular remodeling diseases due to increased MMP activity at sights of arterial remodeling [71, 112], and their activation in response to TNFα in atherosclerosis [52, 151]. However, cathepsin K is regarded as a principle initiators of arterial remodeling pathologies due to their extreme potency for hydrolyzing both insoluble elastin and collagen I [80, 59, 44], as well as their activation in response to hemodynamic shear stress [111].

Although elevated inflammatory cytokines and recruitment of circulating white blood cells aid in the early development of vascular remodeling, hemodynamic shear stress has been regarded for decades as the initiating perturbation to the vasculature to initiate pathological remodeling [73, 152, 49, 96, 31]. Normally, changes in arterial blood flow are detected by the mechanosensitive endothelial cells that line the luminal of the vessel alter vascular tone and maintain a stable wall shear stress. However, sections of the vascular tree that have sharp bends or bifurcations inexorably develop regions of disturbed blood flow, which stimulate pro-remodeling cellular responses in the vascular wall. Specifically, cathepsin K is upregulated at these sites of the disturbed flow, which is secreted into the arterial wall and degrades the elastin fibers that provide mechanical support and function to the arterial wall.

Insoluble elastin fibers in the arterial wall provide the restoring force necessary to propagate blood through large vessels and throughout the body, as well as provide a resistance to blood pressure; loss of elastin integrity leads to reduced blood perfusion and increased risk of vessel rupture, as observed in aortic aneurisms [81, 12]. In addition to the changes in vessel biomechanics, elastin degradation also alters the

phenotypes of the component cells within the arterial wall. Loss of elastin integrity within the medial layer of the artery induces resident smooth muscle cells to revert from a quiescent, contractile state, to a migratory, proliferative state whereby they invade the sub-endothelial space resulting leading to luminal narrowing, further disruption of blood flow, as well as secretion of cathepsin K, ultimately generating a pro-remodeling feed-forward system.

In sickle cell disease, it is believed that regions of disturbed blood flow are not only limited to specific geometries, as is seen with atherosclerosis, but instead can form spontaneously throughout the vascular tree. Sickle red blood cells are inherently adhesive to themselves, other circulating white blood cells, and the vascular endothelium allowing the spontaneous formation of aggregations along luminal side of the vessel wall [61, 64, 20, 40]. While aggregations of circulating red cells are the root of the vasooclusive crises described above, they are not limited to the microvasculature; there is growing evidence that cellular aggregations can spontaneously form in larger vessels resulting in disturbed blood flow [11]. The capacity of the sickle vasculature to spontaneously develop regions of disturbed flow, combined with the ubiquitous presence of elevated TNFα and enhanced mobilization of circulating monocyte, could explain why people with sickle cell disease develop vascular remodeling lesions in vessels, such as the middle cerebral artery, where other remodeling pathologies are rarely seen.

Repetitive sickling of the red blood cell results in premature hemolysis, releasing free heme, the oxygen carrying core of hemoglobin, into the blood plasma [133]. Increased plasma levels of heme scavenge nitric oxide, a powerful vasodilator produced by endothelial cells, crippling arteries capacity to dilate and increase blood profusion. Loss of available nitric oxide by heme scavenging has been hypothesized to specifically hinder vasodilation in the cerebral arteries, not only inhibiting oxygen profusion to the brain, but also may be one of the initiating stages of stroke lesion

development. It is plausible that inhibition of vasodilation could significantly impair endothelial cells capacity to restore hemodynamic shear stress in the presence of a spontaneous red cell aggregation, thereby leading to the premature initiation of arterial remodeling cellular programs and accelerating progression of vascular lesion development. Additionally, loss of nitric oxide has been speculated to result in a hyper sensitivity of the sickle vasculature to vasoconstrictors, such as angiotensin II [9]. Increased angiotensin II signaling is known to increase arterial restructuring akin to what is observed in sickle cell disease, with increased hypertension resulting from intima-media thickening. Furthermore, angiotensin II activates the JNK signaling pathway, which we have shown here to be strongly implicated in arterial remodeling in sickle cell disease (Fig 6-12- 6-14). Therefore, the findings of this study implicate that JNK signaling may represent a significant integration point for the induction of cerebral vascular remodeling in sickle cell disease. We have shown previously that JNK signaling is critical for TNFα induction of cathepsin K in vascular endothelial cells [67, 68], which may contribute to elastin fragmentation stimulating in vivo leading to stimulation of arterial remodeling. Simultaneously, increased reactive oxygen stress may induce angiotensin II-induced signaling of JNK to promote smooth muscle cells proliferation and intimal-medial thickening, and exacerbating arterial remodeling. However, inhibition of JNK signaling, as we have seen in this study reduces cathepsin K activity (Fig 6-13, 6-14), and may also inhibit angiotensin II signaling, thereby mitigating pro-remodeling signals in the arterial wall two-fold.

The development of the Townes sickle transgenic animal model has been an invaluable tool for elucidating the underlying cellular mechanisms that result in the multitude of pathologies and syndromes associated with human sickle cell disease. However, the vasculopathy responsible for development of stroke in sickle cell disease remains largely under-characterized in the transgenic mouse model, hindering robust mechanistic descriptions of how it is initiated and progresses in humans. Townes sickle

transgenic animals have a markedly shorter life span, and are vulnerable to asymptomatic, idiopathic mortality[16, 103]. In this study we are the first to identify that sickle transgenic animals develop substantial remodeling of the carotid arteries, narrowing of the cerebral arteries (Fig 6-4- 6-7), and potentially development of stroke lesions throughout the brain (Fig 6-9), implicating the development of cerebrovascular disease and stroke may also result in the observed decrease in animal survivability. Furthermore, our work here has identified JNK as a potential in vivo integration point for the development of severe arterial remodeling in the carotid and middle cerebral arteries of sickle mice, which has strong implications towards development of pharmaceutical prophylactics for stroke development. Although regular blood transfusions have been shown to mitigate the risk of stroke in young children with sickle cell disease, the chronic transfusions needed carry significant and life-threatening risks of alloimmunity, iron over load, and infection [4, 41, 133]; however, this remains the only available therapy as there are currently no pharmaceutical treatment options available. By identifying other mechanistic pathways by which cathepsin activity is regulated by the hemodynamic and inflammatory environment of sickle cell disease, these may present new therapeutic targets for reduction in the risk of death for young children with sickle cell disease.

CHAPTER VII

FUTURE CONSIDERATIONS

7.1 Major Findings

The work presented in this thesis focused on developing a more complete understanding of the initiation and progression of vascular dysfunction leading to stroke in young children with sickle cell disease. Based on the strong histological similarities between sickle cell vasculopathy and other progressive cardiovascular diseases, specifically rapid remodeling of the cerebral and carotid arteries, we hypothesized that the unique circulatory milieu of sickle cell disease may stimulate upregulation of cathepsins, which have been linked to initiation and progression of arterial remodeling. To achieve this, we systematically investigated how the large artery endothelial cells integrated the complex inflammatory signals unique to circulatory environment of sickle cell disease.

As discussed previously and confirmed by our studies, people with sickle cell disease have chronically elevated serum levels of TNFα, resulting in a perpetual activation of the vascular endothelium. My work demonstrates that TNFα alone is sufficient to induce high levels of cathepsin K and V in large artery endothelial cells, both of which are known to be up-regulated in early arterial remodeling pathologies. Furthermore, I have shown that the circulating monocytes isolated from people with sickle cell disease are more adherent than normal monocytes, and have the unique ability themselves to induce and increase cathepsin K and V, respectively; monocyte-endothelial cell interactions are also argued to be one of the key initiating steps of arterial remodeling. Therefore, the findings from this thesis support the idea that

sickle cell disease promotes and sustains a pro-arterial remodeling environment mediated by elevated cathepsin activation and expression. More specifically, however, I have also identified that both TNFα and sickle monocyte adhesion up-regulate cathepsins K and V through the JNK-c-Jun signaling cascade. Taken together, these data identify three unique targets for novel therapeutic intervention: management of chronic inflammation, direct inhibition of cathepsin K and V activities, and inhibition of JNK/c-Jun activation and signaling.

There is a well-established correlation between the vulnerability of arteries to remodeling, and the magnitude and direction of hemodynamic wall shear stress; specifically, high shear stress is protective from pathological remodeling, and low, oscillatory shear stress promotes remodeling. Furthermore, cathepsin K and V are both known to be up-regulated by the same low, oscillatory shear stress that promotes arterial remodeling. Therefore, given stroke in sickle cell disease results from rapid arterial remodeling, combined with the implication that the remodeling is a result of inflammatory up-regulate cathepsin K and V, it was important that our work also take into consideration the contributions of biomechanical shear stress. Through use of a cone-and-plate shear stress bioreactor, we were capable of actuating physiological shear stress waveforms characteristic of arterial regions protected or vulnerable to remodeling. Of particular note was our conclusion that vasoprotective shear stress is sufficient to significantly reduce active cathepsin K in large artery endothelial cells, even in the presence of TNFα; similarly, pro-remodeling shear stress was able to induce cathepsin K independent of TNFα stimulation. Furthermore, we identified regulation of NFκB expression in arterial endothelial cells as the crucial intracellular signaling cascade responsible for biomechanical regulation of cathepsin K activity. Not only do the findings here serve to directly identify NFκB as another novel therapeutic target, but my work also contributes to a more complete understanding of the fundamental regulation of the systemic and cellular mechanisms that may be responsible for the

generation of arterial occlusion leading to stroke in sickle cell disease.

In an effort to expand these findings into a test system that would allow for greater translation into clinical development of therapies for stroke in sickle cell disease, we investigated the effect of systemic inhibition of JNK signaling in the Townes sickle transgenic mouse model. In our in vivo studies, we identified that sickle cell disease promotes remodeling of the carotid and cerebral arteries, as evidenced by increased elastin fragmentation, delamination, and luminal narrowing, compared to both AA and AS mice. Sickle cell disease also resulted in substantially higher cathepsin K expression in the carotid arteries, suggesting that elevated cathepsin K expression and activity may be linked to the increased incidence of elastin degradation. Additionally, mice with sickle cell disease also presented high neuronal cell death and the development of potential stroke lesions identified by MRI scans. However, administration of the JNK inhibitor SP600125 had the notable result reducing cathepsin expression and activity, as well as reducing the incidence of elastin fragmentation. While preliminary, these results strongly support targeted JNK inhibition as viable therapeutic target for the inhibition of cathepsin K and prevention of sickle cell disease related vasculopathy.

The cumulative findings of this doctoral thesis provide a novel foundation for understanding the dynamic interactions between the unique inflammatory and hemodynamic environment in sickle cell disease, and their contributions to promoting the formation of stroke in young children. Through the use *in vitro* bioreactor systems, my work has identified multiple new therapeutic targets and begun to test their validity in in vivo animal models. However, there remains a large body of work yet to be completed with regards to more completely understanding the hemodynamic environment of the cerebral vasculature, and its potential effect on cathepsin-mediated arterial remodeling, the incorporation of more advanced analysis techniques for more complete understanding of the biomolecular networks involved, and development and

implementation of more advanced *in vitro* culture technologies.

7.2 Exploration of cathepsin K activation in cerebral vasculature through in vitro actuation of cerebral blood flow

Work discussed in this thesis has underscored the critical regulatory nature of hemodynamics in regulating the structure and function of the vasculature, and how alterations to wall shear stress can generate powerful feed-forward loops that lead to pathological arterial remodeling, potentially leading to stroke in children with sickle cell disease. However, the sickle mutation also significantly changes the bulk biophysical properties of whole blood. The altered red blood cell morphology caused by sickling, combined with aberrant aggregation of red cells to each other, circulating white blood cells, and the arterial wall result in changes in blood viscosity in non-intuitive ways, potentially leading to unique hemodynamic shear stresses that develop through the vessels known clinically to be vulnerable to stroke formation. By analyzing whole blood obtained from donors with sickle cell disease, it would be possible to obtain key, bulk physical properties, such as viscosity, that could then be incorporated into a computational fluid dynamic model of the vasculature to directly calculate pathophysiologically relevant wall shear stress unique to sickle cell disease at several points along the vascular tree. Since alterations in hemodynamic shear stress have long been regarded as a key initiating factor of pathological arterial remodeling, it would be highly advantageous to incorporate the unique biophysical properties of the hemodynamic environment in the study of sickle cell related vasculopathy.

Work done by the Veneziani group at Emory University has developed advanced computational tools to recreate three-dimensional, arterial geometries through analysis of magnetic resonance angiography (MRA) images [6, 109]. The reconstructed geometry can then be imported into novel computational fluid dynamic software, also

developed by the Veneziani group, that develops a high resolution, finite element analysis (FEA) model of the vasculature that can be used to calculate multiple system values, including wall shear stress, blood pressure, and multi-dimensional flow profiles with high spatiotemporal fidelity based on a small number of user-defined boundary and initial conditions. The ability to analyze human arterial geometries is of considerable benefit as it will allow for an unprecedented level of comparison of the changes in the circulatory and hemodynamic environment in sickle cell disease compared to people with normal hemoglobin, especially in the regions of the cerebral vasculature where little is known about hemodynamic flow profiles. Use of advanced computational fluid analysis can also be easily paired with the *in vitro* cone-and-plate shear system used in our lab. Derivation of the time-dependent wall shear stress could be input into the shear bioreactor and actuated on arterial endothelial cell cultures with the for millisecond temporal accuracy. Additionally, this technology could then be used to analyze unique differences in vasculature and hemodynamics between people without sickle cell disease, people with no history of sickle cell related stroke, those at risk for sickle related stroke, and those with a history of stroke. Currently, there the only predictive method for identification of stroke risk in children with sickle cell disease is transcranial Doppler ultrasound (TCD), in high risk is defined as blood velocities greater than 200cm/s [3, 60, 2]. While non-invasive and useful for ascertaining relative risk of stroke, TCD generally does not have sufficient resolution to evaluate the severity or location of stroke lesions. However, more complete mechanistic investigations into potential differences in vascular geometry, and biophysical properties of blood from a wide range of patients with varying stroke risk could lead to novel discoveries and more accurate predictive and preventative methods. Additionally, bi-directional investigations between computation analysis and *in vitro* bioreactor experiments would also allow for novel retrospective and prospective clinical studies. MRAs taken from patients with identified sites of arterial remodeling, leading

to stroke, could be analyzed through computational analysis to determine hemodynamic shear profiles near the site of occlusion, actuate those shear stresses on arterial endothelial cells, and quantify the resulting changes in cathepsin activity, and intracellular kinases which could be used as novel predictive biomarkers for early detection of arterial remodeling. Correlation of cathepsin upregulation activity, in conjunction with up-stream kinase signals, with severity of stroke progression could also lead to the development of novel preventative measures, by which physicians could more accurately assess the risk a given patient has for the development of stroke as well as provide more accurate and personalized treatment options.

7.3 Development of high resolution imaging of vascular remodeling proximal to stroke lesions in sickle transgenic mice

The development of the sickle transgenic mouse with humanized hemoglobin has been a substantial boon to the entire field dedicated to understanding the *in vivo* repercussions of sickle cell disease; however many of the human pathologies associated with sickle cell disease are not yet characterized in the animal model. This study was not only the first to describe the development of cathepsin-mediated vascular remodeling in the carotid and cerebral arteries in sickle transgenic animals comparable to what is observed in humans, but was also the first study to identify the potential development of stroke lesions. While the findings from this study are extremely promising for developing a more complete, mechanistic understanding of the initiation, progression, and regulation of sickle mediated arterial remodeling, additional investigations must be completed.

Stroke lesions were identified in these studies through the use of high-resolution MRI scanning to identify areas of the brain that developed a dark appearance on the image, indicative of poor blood profusion and possible stroke development. The brains should then be histologically processed to identify morphological changes in

the cerebral arteries around the anomalous regions identified by MRI. By cross validating the MRI imaging with histological morphology, it could be more conclusively stated how the stroke lesions developed, better characterize the type of lesion (hemorrhagic versus ischemic), and characterize similarities between the pathology of stroke in mice compared to what is known about sickle cell mediated stroke in human patients. However, this process is laborious and would require an extremely high level of histological proficiency to successfully reconstruction of the entire brain vasculature for a 1:1 comparison to the MRI scans. Additionally, the high degree of tortuosity of the cerebral vessels would make it difficult to predict the axis on which to conduct the histological slices; based on which surgical axis the vessel is oriented; sectioning along the incorrect axis significantly impair the ability to identify evidence of arterial remodeling and other morphological changes in and around the stroke lesion. To address these complications and limitations, one could use plastination of the cerebral vessels to gain high resolution reconstruction of changes in the arterial network as a result of sickle cell disease.

The cerebral vessels in mice are substantially smaller than those found in humans, and therefore it is difficult to obtain high resolution, structural images of the vessels using traditional, non-invasive techniques such as MRA or angiography. However, micro computed tomography (μCT) is capable of generating high resolution imaging of internal vasculature of small animals, such as mice. Perfusion of a liquid plastic polymer throughout the vasculature of the animal perimortem allows for the delivery of a contrast agent that is detected by the μCT imager; the plastic solidifies shortly after profusion preventing diffusion or weakening of the contrast signal. Three-dimensional reconstructions of the cerebral vasculature can be analyzed on a population level to identify systemic changes in the profusion of the plastic that could indicate regions of arterial narrowing or occlusion in sickle animals. The plastinated brains could then be imaged by MRI to determine if potential stroke lesions co-localize with the identified

regions of arterial narrowing. Additionally, the reconstructed μCT scans could also be used to generate finite element models of cerebral blood flow in the transgenic animal models, as we discussed previously. Analysis of blood velocity, wall shear stress, and blood viscosity and generation of predictive models for the development of stroke lesions in the transgenic animals, compared to human data could further validate the use of the Townes mouse model for the study of sickle cell related vascular disease.

7.4 in vivo investigation of specific cathepsin K inhibitors as novel drug therapeutics for prevention of stroke in sickle cell disease

The finding sickle transgenic animals had substantially elevated cathepsin K expression in the carotid arteries, which co-localized with the increased degradation of the elastin fibers within the arterial wall strongly supports the hypothesis that sickle cell mediated arterial remodeling is propagated, to a significant level, by the pathological activation of cathepsin K. In this study, inhibition of JNK was shown to be effective in decreasing cathepsin K expression and activity in the sickle vasculature, as well as preventing arterial degradation, based on our findings from *in vitro* studies, JNK signaling is strongly implicated in activation of cathepsin K in vascular endothelial cells. However, JNK signaling is critical in several cellular responses to inflammation, damage, and infection; therefore, systemic inhibition of JNK signaling may have substantial side effects in humans, although none were observed in the mice. However, direct, targeted inhibition of cathepsin K could prove to be highly effective in the prevention of sickle cell related vasculopathy. Odanacatib is a highly specific cathepsin K specific inhibitor that is currently undergoing FDA approval for the treatment of osteoporosis and bone metastasis[45, 33]. Initiation of animal trials for odanacatib would not only provide additional verification that pathological cathepsin K activation in sickle cell disease leads to increased vascular remodeling, but may also identify odanacatib as the first ever drug for the safe treatment and prevention of vascular

remodeling and potentially stroke development in sickle cell disease.

7.5 Application of systems biology for analysis of heterogeneity of vascular pathology sickle cell disease

In conjunction with the influence of hemodynamic shear stress initiation of arterial remodeling, findings from my *in vitro* studies implicate chronic exposure to inflammatory cytokines, such as TNFα, as well as increased monocyte-endothelial cell interactions, are potent activators of cathepsin activity in arterial endothelial cells, particularly in sickle cell vasculopathy. However, while all people who suffer from sickle cell disease possess the same genetic mutation, clinical case studies of multiple sickle cell pathologies report a wide dichotomy of severity among different patients; sickle cell disease increases risk of stroke in young children over 200-fold, but stroke is only reported in about 10% of affected children. Some studies attribute decreased severity in some patients due to a natural preservation of the expression of fetal hemoglobin (HbF). Similar to -globin, HbF forms a tetramer with β-globin molecules to form a complete hemoglobin molecule, however, human expression of HbF is usually silenced and replaced by -globin within weeks after birth. However, some people with sickle cell disease retain significant levels of HbF expression well into adulthood, effectively diluting the level of expressed sickle hemoglobin. Reactivation of HbF is also the hypothesized mode of action of hydroxyurea, the only pharmacological treatment shown to be effective at ameliorating complications of sickle cell disease; however, hydroxyurea alone has not been effective at reducing the incidence of strokes in young children.

Research by Park et al. and others [101, 15] successfully demonstrated that systems biology can be applied to generate predictive models of disease outcomes based on analysis of a variety of biomarkers and signaling kinases. Furthermore, systems biology is unique in its ability to process extremely large data sets to determine which

inputs co-vary with known pathologies as highly efficient way to parse out how complex systems are regulated and influence clinical outcomes in otherwise unforeseen ways. In sickle cell disease, patients can have variations in serum levels of TNFα and other inflammatory cytokines, numbers of circulating monocytes, relative cathepsin activity levels, red blood cell population composition, fetal hemoglobin content, and a variety of other factors that could themselves, or in conjunction with other factors, modulate a person's susceptibility to comorbidities, such as stroke development. Many of these values may also vary depending if the person is experiencing a vasoocclusive crisis. The number of variables that can contribute to sickle cell pathologies make systematic investigation and development of comprehensive, mechanistic explanations for the development and progression of known pathologies difficult.

Sickle cell disease can be thought of as a disease of compounding unity: a mutation in nucleotide of one gene that results in one amino acid substitution in one protein expressed in only one type of cell. Since the cells most directly affected by the sickle mutation are circulating red blood cells, every organ system in the body is negatively effected in an appreciable, and sometimes, deadly ways. However, investigations into sickle cell disease often operate in relative isolation due to somewhat arbitrary divisions; it is rare to see investigations on a multi system scale. This tendency towards isolative investigation is an artifact of needing to reduce the number of variables in the system, but the resulting assumptions preclude the possibility that complications from one sickle pathology could have a significant impact on other pathologies. Using a systems biology approach, it would be possible to use efficiently and accurately quantify hundreds, maybe thousands, of data points across multiple cell and tissue types.

Chronic inflammation is a serious syndrome associated with sickle cell disease, and has been implicated here as an initiating factor in arterial vasculopathy and stroke development in young children. However the perpetuation of inflammation

in sickle cell disease is contested by several investigators, some hypothesizing that chronic inflammation is the result of pathological adherence of red blood cells to the vascular endothelium, resulting in systemic endothelial damage and dysfunction. Others contend that chronic inflammation may result from ischemic-profusion injury and exposure to reactive oxygen species generated by repetitive vasooclusive crises in the microvasculature. The work presented here, and research conducted by others [67, 114, 40], indicate that circulating monocytes in sickle cell disease are pathologically activated to be more adherent to the endothelium and secrete 300% higher amounts of TNFα [14]. It is likely that there exists one single event that, alone, generates and propagates the chronic inflammatory environment in sickle cell disease, but expansion of experimental designs to incorporate multiple cell types stimulated with different chemical and biophysical conditions would allow for the generation of a more robust and physiologically relevant *in vitro* model system. Advances in multiplex kinase, cytokine, protein, and genetic assays allow for thousands of different biomolecules to be assayed and quantified simultaneously, resulting in the generation of a comprehensive molecular networks. Use of similar experimental techniques has shown to be effective in the study of endometriosis, another inflammatory disease characterized by heterogeneity in system presentation and severity, as well as variability in clinical outcomes. Researchers were capable of precisely identifying not only which immune cells significantly influence the severity of endometrial lesion growth in an *in vivo* animal model, but also which specific signaling pathways within those immune cells could most effectively be targeted for maximal therapeutic effect using advanced multiplex experimental systems in conjunction with systems biology techniques.

APPENDIX A

CODE FOR ACTUATION OF PHYSIOLOGICAL WAVEFORMS IN CONE-AND-PLATE BIOREACTOR

```
1  PROGRAM
2  'Program 0
3  'TODO: edit your program here
4  CLEAR
5  drive on A
6  drive on B
7  drive on C
8  drive on D
9  drive on V
10 drive on X
11 drive on Y
12 drive on Z
13
14 dim LV(1)
15
16 #DEFINE timecount LV0
17 #DEFINE sysclock P6916
18
19 sysclock = 0
20 timecount = 0
21
```

```
22 while (timecount<100000)
23
24  sysclock = 0
25
26
27  while (sysclock<30)
28   JOG ACC A 100 B100 V100 X100 C80 D80 Y80 Z80
29   JOG VEL A2.77 B2.77 V2.77 X2.77 C2 D2 Y2 Z2
30   JOG FWD A B C D V X Y Z
31  wend
32
33  while ((sysclock=30) OR (sysclock>30) AND (sysclock<60))
34   JOG ACC A110 B110 V110 X110
35   JOG DEC C91 D91 Y91 Z91
36   JOG VEL A2.77 B2.77 V2.77 X2.77
37   JOG VEL C0 D0 Y0 Z0
38   JOG FWD A B V X
39   JOG FWD C D Y Z
40  wend
41
42  while ((sysclock=60) OR (sysclock>60) AND (sysclock<95))
43   JOG ACC A110 B110 V110 X110
44   JOG ACC C125 D125 Y125 Z125
45   JOG VEL A2.77 B2.77  V2.77 X2.77
46   JOG VEL C4.15 D4.15 Y4.15 Z4.15
47   JOG FWD A B V X
48   JOG REV C D Y Z
```

```
49  wend
50
51  while ((sysclock=95) OR (sysclock>95) AND (sysclock<115))
52   JOG ACC A110 B110 V110 X110
53   JOG DEC C101 D101 Y101 Z101
54   JOG VEL A2.77 B2.77 V2.77 X2.77
55   JOG VEL C0 D0 Y0 Z0
56   JOG FWD A B V X
57   JOG REV C D Y Z
58  wend
59
60  while ((sysclock=115) OR (sysclock>115) AND (sysclock<145))
61   JOG ACC A115 B115 V115 X115
62   JOG DEC C101 D101 Y101 Z101
63   JOG VEL A8.33 B8.33 V8.33 X8.33
64   JOG VEL C0 D0 Y0 Z0
65   JOG FWD A B C D V X Y Z
66  wend
67
68  while ((sysclock=145) OR (sysclock>145) AND (sysclock<170))
69   JOG ACC A115 B115 V115 X115
70   JOG ACC C58 D58 Y58 Z58
71   JOG VEL A8.33 B8.33 V8.33 X8.33
72   JOG VEL A0.78 B0.78 Y0.78 Z0.78
73   JOG FWD A B V X
74   JOG FWD C D Y Z
75  wend
```

```
76
77  while ((sysclock=170) OR (sysclock>170) AND (sysclock<195))
78   JOG ACC A115 B115 V115 X115
79   JOG DEC C30 D30 Y30 Z30
80   JOG VEL A8.33 B8.33 V8.33 X8.33 C0.01 D0.01 Y0.01 Z0.01
81   JOG FWD A B V X
82   JOG FWD C D Y Z
83  wend
84
85  while ((sysclock=195) OR (sysclock>195) AND (sysclock<200))
86   JOG DEC A26 B26 V26 X26
87   JOG DEC C30 D30 Y30 Z30
88   JOG VEL A3.5 B3.5 V3.5 X3.5
89   JOG REV C0.01 D0.01 Y0.01 Z0.01
90   JOG FWD A B V X
91   JOG FWD C D Y Z
92  wend
93
94  while ((sysclock=220) OR (sysclock>200) AND (sysclock<260))
95   JOG DEC A26 B26 V26 X26
96   JOG ACC C19 D19 Y19 Z19
97   JOG VEL A3.5 B3.5 V3.5 X3.5 C0.97 D0.97 Y0.97 Z0.97
98   JOG FWD A B V X
99   JOG REV C D Y Z
100 wend
101
102 while ((sysclock=260) OR (sysclock>260) AND (sysclock<305))
```

```
103   JOG DEC A26 B26 V26 X26
104   JOG ACC C27 D27 Y27 Z27
105   JOG VEL A3.5 B3.5 V3.5 X3.5
106   JOG VEL C0 D0 Y0 Z0
107   JOG FWD A B V X
108   JOG REV C D Y Z
109  wend
110
111  while ((sysclock=305) OR (sysclock>305) AND (sysclock<380))
112   JOG DEC A26 B26 V26 X26
113   JOG ACC C13 D13 Y13 Z13
114   JOG VEL A3.5 B3.5 V3.5 X3.5
115   JOG VEL C0.83 D0.83 Y0.83 Z0.83
116   JOG FWD A B V X
117   JOG FWD C D Y Z
118  wend
119
120  while ((sysclock=380) OR (sysclock>380) AND (sysclock<400))
121   JOG DEC A26 B26 V26 X26
122   JOG DEC C11 D11 Y11 Z11
123   JOG VEL A3.5 B3.5 V3.5 X3.5
124   JOG VEL C0 D0 Y0 Z0
125   JOG FWD A B V X
126   JOG FWD C D Y Z
127  wend
128
129  while ((sysclock=400) OR (sysclock>400) AND (sysclock<465))
```

```
130   JOG ACC A25 B25 V25 X25
131   JOG DEC C11 D11 Y11 Z11
132   JOG VEL A3.82 B3.82 V3.82 X3.82
133   JOG VEL C0 D0 Y0 Z0
134   JOG FWD A B V X
135   JOG FWD C D Y Z
136  wend
137
138  while ((sysclock=465) OR (sysclock>465) AND (sysclock<510))
139   JOG ACC A25 B25 V25 X25
140   JOG ACC C3 D3 Y3 Z3
141   JOG VEL A3.82 B3.82 V3.82 X3.82
142   JOG VEL C0.5 D0.5 Y0.5 Z0.5
143   JOG FWD A B V X
144   JOG FWD C D Y Z
145  wend
146
147  while ((sysclock=510) OR (sysclock>510) AND (sysclock<610))
148   JOG ACC A16 B16 V16 X16
149   JOG ACC C3 D3 Y3 Z3
150   JOG VEL A3.02 B3.02 V3.02 X3.02
151   JOG VEL C0.5 D0.5 Y0.5 Z0.5
152   JOG FWD A B V X
153   JOG FWD C D Y Z
154  wend
155
156  while ((sysclock=610) OR (sysclock>610) AND (sysclock<745))
```

```
157    JOG DEC A4 B4 V4 X4
158    JOG ACC C3 D3 Y3 Z3
159    JOG VEL A2.63 B2.63 V2.63 X2.63
160    JOG VEL C0.5 D0.5 Y0.5 Z0.5
161    JOG FWD A B V X
162    JOG FWD C D Y Z
163   wend
164
165   while ((sysclock=745) OR (sysclock>745) AND (sysclock<975))
166    JOG DEC A4 B4 V4 X4
167    JOG DEC C1.9 D1.9 Y1.9 Z1.9
168    JOG VEL A2.63 B2.63 V2.63 X2.63
169    JOG VEL C0.17 D0.17 Y0.17 Z0.17
170    JOG FWD A B V X
171    JOG FWD C D Y Z
172   wend
173
174   timecount = timecount +1
175
176 print "Timecount=", timecount
177 print "Sysclock=", sysclock
178 wend
179
180 jog off A B C D
181
182 ENDP
```

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