Specific Aims:

Sickle cell disease (SCD) is a common and life-threatening autosomal recessive hematological disorder that affects almost 100,000 people in the US and millions worldwide. Abnormal hemoglobin (Hb) production greatly shortens the lifespan of red blood cells (RBCs) that upon deoxygenation sickle, dehydrate, become less deformable and abnormally adhesive. Repeated sickling and chronic hemolytic anemia ultimately results in substantial morbidity and early mortality in these patients. Amongst acute complications in SCD, acute chest syndrome (ACS) is a leading cause of hospitalization and the most common cause of death due to SCD. Since the pathophysiology underlying ACS is still unclear, current supportive interventions include oxygen therapy, antibiotics and RBC transfusions.

Our long-term objective is to characterize key factors that play a role in ACS pathogenesis in order to identify targets that can be used to more effectively treat ACS. *Our overarching hypothesis is that alternative complement pathway (ACP) activation contributes to hemolysis and the pathogenesis of ACS in SCD.* Our hypothesis is formulated on the basis of our recent discovery that injection of cobra venom factor (CVF), a commonly used approach to induce complement activation, results in acute hemolysis and death in sickle (SS) mice. Consistent with clinical ACS, death in these mice was preceded by rapid deoxygenation, hypopnea and bradycardia (all hallmarks of acute lung injury (ALI) in mice). In contrast, CVF treated littermate control (AA) mice did not develop detectable hemolysis, pulmonary compromise or increase in mortality. In addition, preliminary data from a retrospective study analyzing a cohort of children during ACS and at baseline 4 weeks after ACS resolution demonstrates that complement components 3a (C3a) and 5a (C5a), as well as other markers of proximal ACP activation were consistently increased during ACS compared to baseline; strongly suggesting that ACP activation occurred during ACS. While CVF-mediated ALI in SS mice could induce endothelial activation, neutrophil accumulation and anaphylatoxin mediated ALI, our preliminary data demonstrate that CVF treated SS mice had markedly increased plasma free hemoglobin consistent with intravascular hemolysis, while the control mice did not. Consistent with the potential role of acute hemolysis in ACS pathogenesis, our clinical preliminary data demonstrate that significant hemolysis occurred in the vast majority of patients with ACS, with a drop-in hemoglobin (Hb) between 2-3 g/dL from their baseline. As ACP activation can be triggered by free heme[1], exuberant activation of complement may contribute to intravascular hemolysis during ACS, which would be predicted to result in the release of additional free heme, further complement activation and ultimately exacerbation of hemolysis. Rapid drops in Hb, release of free heme and production of C3a/C5a would be predicted to facilitate neutrophil activation and endothelial activation/injury, ultimately leading to ACS. However, while complement activation represents a potentially pharmacologically targetable process, no studies to date have directly defined the role of complement in the development of ACS. To test our central hypothesis, we will pursue the following specific aim:

<u>Aim 1</u>: Define the role of complement mediated neutrophil activation and endothelial injury in the development of ALI in SS mice. We will use our *in vivo* model of CVF-mediated complement activation to test our hypothesis that free-heme and complement work in concert to trigger neutrophil activation, endothelial injury and ALI development in SS mice.

Successful completion of these studies will define the key involvement of ACP in ACS, and more importantly could lead to a paradigm shift in the mechanism(s) of hemolysis in SCD pathophysiology. These studies may also provide an important framework to develop rational approaches to prophylactically predict, prevent or even abort ACS, and to mitigate hemolysis-mediated organ injury. This JFF award will enable me to receive training in mechanistic studies and plan in modeling of pharmacologic inhibitors for ACP in SCD. The results and experience gathered from this work will position me to be a competitive candidate for a NIH K08 application.

Significance and Premise. SCD affects about 300,000 births annually and 3 million Americans. ACS is a common and serious complication of SCD.[2]

A. Medical Significance of Acute Chest Syndrome:

ACS is associated with high morbidity and mortality: The re-hospitalization rate after the initial ACS in children <4 years of age is 62% in the 1st year.[3] ACS is the leading cause of intensive care unit admission[4] and accounts for as many as 25% of deaths in SCD.[5]

ACS is associated with hemolysis: Reticulocytosis and elevated LDH are often seen with severe SCD and ACS[6] consistent with our preliminary data of ~ 2 g/dL drop in Hb during ACS. Elevated levels of plasma free heme can induce pro-inflammatory responses, directly activate the endothelium and facilitate complement activation, likely contributing to SCD pathogenesis.

ACS is associated with multi-organ failure (MOF): Rapidly progressive ACS is associated with hemolysis, thrombocytopenia, acute kidney injury, altered mental status, MOF and death; features consistent with atypical hemolytic uremic syndrome (aHUS), a complement-mediated disease.[7] These data suggest a key role for free heme and complement in ACS pathogenesis.

B. Scientific premise:

No current pharmacological targets exist to prevent or mitigate ACS: Current clinical management of ACS is entirely supportive care. This study will explore the role of free heme and complement in ACS to potentially develop a biomarker to predict, prevent or treat ACS.

Lack of understanding on the predictors of ACS in SCD: While increased incidence of asthma and allergic inflammation is found in SCD, there is no clear mechanism or prognostic indicators for ACS development. C3a/C5a are known to be potent pro-inflammatory mediators in asthma, suggesting a potential link between ACP and ACS.[8]

Contribution of ACP in the pathophysiology of ACS and development of a therapeutic strategy: Microangiopathic hemolysis with ACP activation in SCD patients with vaso-occlusive crisis (VOC) and ACS have been reported, including our report on successful use of eculizumab, a monoclonal antibody against C5, strongly suggesting ACP role in ACS pathogenesis.[9,10,11]

C. The key contribution of this proposal will be characterizing the role of ACP in hemolysis and ACS in pre-clinical models: Successful completion of proposed studies will further our understanding of ACS pathogenesis, allow us to develop diagnostic tools that can help predict the likelihood, and identify key therapeutic targets to prevent or abort ACS.

Innovation. Patients with SCD and ACS are managed only by supportive care due to lack of disease modifying agents. Given our preliminary data, employing agents that deliberately alter ACP may offer novel approaches to predict, prevent or relieve ACS.

Novel hypothesis focused on defining the key involvement of ACP in ACS pathogenesis: Studies performed during ACS episode demonstrate significantly increased C3a/C5a. Our pre-clinical model also corroborates these results, providing a novel system to examine ACP in ACS.

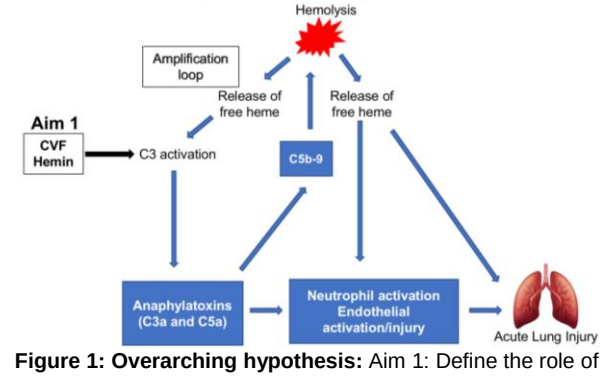


Figure 1: Overarching hypothesis: Aim 1: Define the role of ACP mediated neutrophil activation, endothelial activation and injury in the development of ALI in mice with SCD.

Unique murine models of complement-mediated ALI in humanized SS mice with and without global C3 deletion: These robust and reproducible models will help identify critical factors that regulate ACP activation, including the impact of heme on ACP and neutrophil activation, endothelial injury and additional hemolysis. Identification of these factors will also enable us to test complement inhibitors on the development and treatment of ALI in SS mice.

Use of novel biomarkers to test for ACP activation in SCD: Previous studies have mostly used total complement levels, while we will be analyzing a comprehensive set of ACP activation products: C3a/C5a, C5b-9 and Ba/Bb. There is currently no literature on these markers in SCD mice or humans, except for our published report[11] and preliminary data. These studies possess the capacity to **identify for the first-time the role of ACP in ACS, and may identify targets that predict and prevent ACS**.

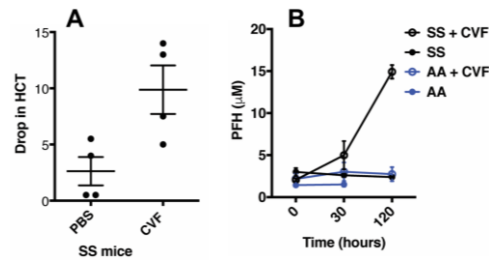


Figure 2. Estimation of hematocrit (HCT) and plasma free Hb (PFH). A: Drop in HCT from baseline in PBS or CVF treated SS mice. B: PFH levels in PBS or CVF treated SS or AA mice.

Approach (includes background and preliminary data).

Involvement of ACP in SCD - Historically SCD patient's susceptibility to pneumococcal infection was ascribed to low levels in one or more complement proteins,[12] which was subsequently found to be secondary to consumption from ACP activation.[13,14] Additional explanations for susceptibility of sickle RBCs to ACP activation include increased PE/PS exposure on deoxygenated sickle RBCs,[15] crosstalk between thrombin and complement,[16,17] and direct activation of C3 by heme in serum and on endothelial cells.[1]

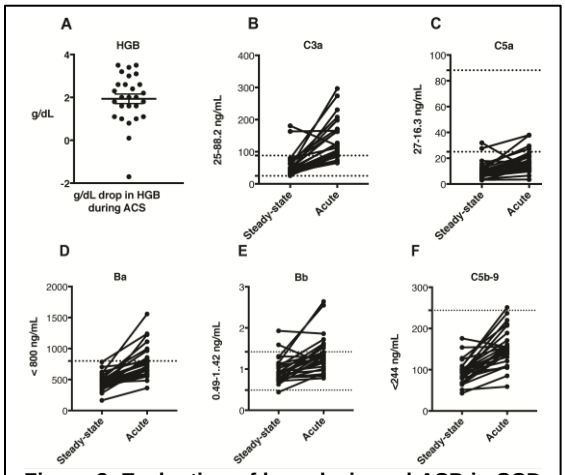


Figure 3. Evaluation of hemolysis and ACP in SCD patients presenting with ACS. Fall in Hb during ACS (A). Complement proteins C3a, C5a, Ba, Bb and C5b-9 (B-F) were analyzed during 'Acute' crisis and 'Steady-state'. Dotted lines signify normal range.

Complement-mediated intravascular hemolysis in SCD humans and mice – Our preliminary data demonstrate that SS mice injected with CVF experience a marked drop in hematocrit and increase in plasma free Hb from baseline to 30 min post treatment, compared to AA mice (Fig. 2). Consistent with this, 27-paired stored plasma samples from patients during ACS ('acute') and 4 weeks later ('steady-state') demonstrate hemolysis in the vast majority of patients, with a 2-3 g/dL drop in Hb (Fig. 3A), which is in parallel with a prior study.[6] These results suggest that significant intravascular hemolysis occurred during ACS.

Evidence of ACP activation during ACS in SCD - In the above cohort, C3a/C5a and Ba/Bb were increased during ACS in comparison to their own baseline, strongly suggesting that ACP activation occurred during ACS (Fig. 3B-E). C5b-9, though increased from baseline remained within the normal range (Fig. 3F), suggesting that a 'full-blown' aHUS was averted due to the presence of complement inhibitory proteins. This represents the first study of its kind to provide detailed evaluation of ACP activation in SCD patients with ACS.

Complement activation in SS mice results in ALI - SS and AA mice were treated initially with PBS to establish a baseline, followed by intraperitoneal injection of CVF. SS mice displayed rapid deoxygenation, bradycardia and hypopnea (Fig. 4A-C) consistent with ALI. The wet to dry lung weight ratio was significantly increased in SS mice (Fig. 5). Lung histopathology revealed neutrophil accumulation, edema, occasional areas of hemorrhage in parenchymal and vascular spaces (Fig. 5). We discovered that CVF treatment (tolerated without symptoms in AA mice) is rapidly fatal to all SS mice (Fig. 6). With these data, we postulate that fatality of SS mice is from ACP mediated anaphylatoxins and free-heme working in concert to trigger neutrophil accumulation, endothelial activation and injury, as shown in prior models of ALI.[18,19]

<u>Aim 1</u>: Define the role of complement-mediated neutrophil activation and endothelial injury in the development of ALI in SS mice. Rationale: Our pre-clinical model suggests that ACP activation results in the development of ALI in SS mice. Similar to our clinical data, the release of C3a/C5a may represent a key process in our pre-clinical model of ACP-mediated ALI. C5a, activates neutrophils,[19] which has been shown to directly induce ALI.[20] This suggests a fundamental role of ACP in the development of ALI in SS mice. We did not see any evidence for other organ/s injury in these SS mice (data not shown). In addition, C3a/C5a possess the ability to directly activate endothelial cells,[21,22] which may also facilitate pulmonary neutrophil entrapment. Furthermore, complement-mediated hemolysis of sickle RBCs likely results in the rapid release of Hb, causing significant increases in free heme. As heme itself can activate complement[23] and is a potent activator of endothelial cells,[24] ACP-mediated hemolysis may result in a positive feedback loop that can further increase endothelial activation, neutrophil retention and activation, endothelial injury and ultimately ALI (Fig 1). Thus, ***we hypothesize that ACP-induced hemolysis of sickle RBCs results in rapid increases in free Hb/heme, which works in concert with complement byproducts to facilitate endothelial, neutrophil and further ACP activation that in turn results in endothelial damage and ALI.*** To examine this, we propose the following approach:

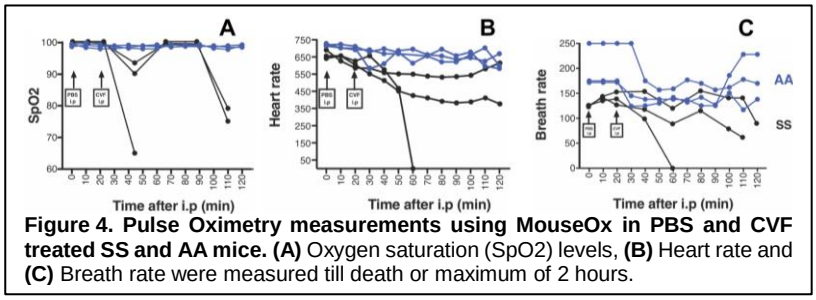
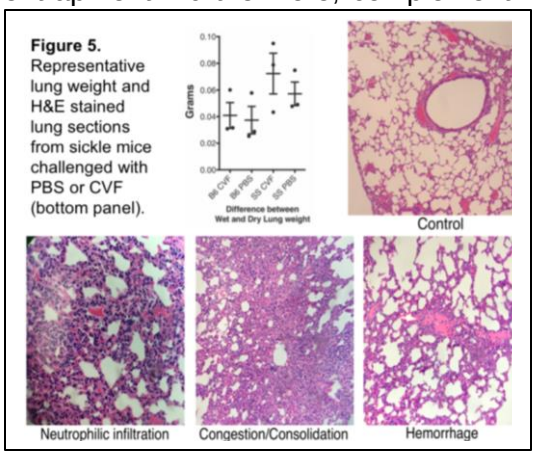


Figure 4. Pulse Oximetry measurements using MouseOx in PBS and CVF treated SS and AA mice. (A) Oxygen saturation (SpO2) levels, **(B)** Heart rate and **(C)** Breath rate were measured till death or maximum of 2 hours.



<u>Aim 1A: Define the role of complement-mediated neutrophil activation on ALI in SS mice</u>. To study this *in vivo*, we will first examine CVF treated SS and AA mice for plasma ACP products (Ba, Bb, C3a, C5a and C5b-9 levels using ELISA kits). In addition, we will measure the presence of free heme to correlate with ACP. As neutrophil activation can result in extracellular trap (NET) formation and NETs not only facilitate neutrophil injury, but detected in lungs following the development of ACS,[25] we will assess NET formation in the peripheral blood and lungs of CVF treated mice. Briefly, neutrophils isolated from PBMCs will be stained with both cell-impermeable Sytox orange and cell-permeable SYTO13, and then imaged by confocal microscopy for the presence of NETs (if DNA fiber length exceeds 50µm). To examine for NETs in the lungs, mice will be injected with an anti-CD31 antibody for *in vivo* staining of lung endothelial cells 15 min prior to euthanasia. Lungs will be fixed, sectioned, and then stained with Sytox orange for imaging.[26,27] Additionally, neutrophils isolated from untreated SS and AA mice will be treated with C5a *in vitro* to define whether C5a can also induce NET formation.[27] To define the role of complement in NET formation *in vivo*, we will inject hemin (known to cause ALI in mice) into (SS x C3 KO) mice and assess NET formation as described above. Finally, to specifically test the role of neutrophils in the development of ALI, we will deplete neutrophils in

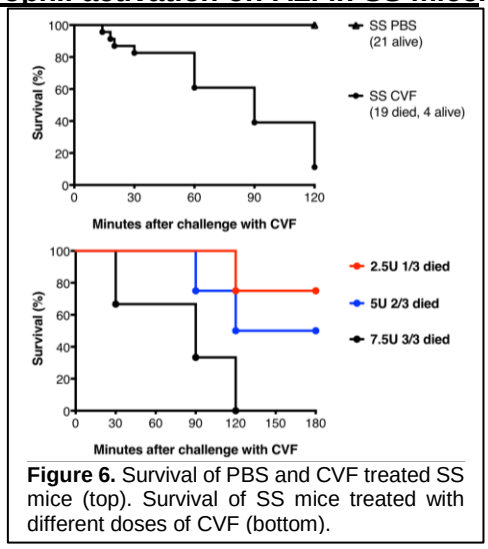


Figure 6. Survival of PBS and CVF treated SS mice (top). Survival of SS mice treated with different doses of CVF (bottom).

mice using anti-Gr1 monoclonal antibody (clone: RB6-8C5), followed by CVF or hemin injection. In each experiment, the potential impact of CVF or hemin on pulmonary function will be assessed using MouseOx. In addition, neutrophil plugging, endothelial cell gap and bleb formation suggestive of endothelial injury, interstitial edema and intra-alveolar hemorrhage will be scrutinized on lung sections.

Aim 1B. Define the role of complement on endothelial activation and injury in SS mice. To initially evaluate endothelial activation, plasma samples obtained at 10 min, 30 min, 1 h and 2 h following CVF treatment of SS or AA mice will be evaluated for soluble endothelial activation markers (P-selectin, VCAM, and ICAM1). To more directly evaluate endothelial activation *in situ*, lungs harvested at each time point will be evaluated for endothelial P-selectin expression, as described previously.[28] To define the role of complement in hemin-induced ALI, we will similarly evaluate levels of soluble endothelial activation markers and the expression of endothelial adhesion molecules *in situ* following injection of hemin into (SS x C3 KO) mice. To define the role of CVF or hemin induced endothelial activation on neutrophil entrapment and activation, 100 mg/kg of a small molecule antagonist of P-selectin (PSI-697, 100mg/kg) known to prevent neutrophil rolling and recruitment, will be injected prior to CVF or hemin injection. Alexa-488 anti-Ly-6G antibody will be used to quantify adherent neutrophils in comparison. Neutrophil accumulation and NET formation (as described in Aim 1A) will be assessed in blood and lung tissues. The potential role of neutrophil adhesion following hemin-induced ACP activation will additionally be assessed using a similar approach in (SS x C3 KO) mice. Finally, to define the role of neutrophils on endothelial activation and injury, neutrophil will be depleted (as described in Aim 1A), followed by similar evaluation for endothelial activation/ injury. In addition to lung histopathology for edema and hemorrhage, endothelial dysfunction will be evaluated by examining vascular permeability, RBC adhesion, enhanced oxidant stress, Weibel-Palade body release of von Willebrand factor and P-selectin expression.

Aim 1 expected outcomes and alternative approaches - Following CVF administration, we anticipate seeing elevated C3a/C5a and C5b-9 levels that will correlate with plasma free Hb/heme, suggesting the ability of ACP to induce hemolysis in SS mice. We also expect to observe increased NET formation in the peripheral blood and lungs of CVF or hemin treated SS mice. We anticipate that NETs will co-localize with complement byproducts, suggestive of ACP activation in the vicinity of neutrophil activation and NETosis. While we anticipate that CVF will result in ACP activation, given the ability of neutrophils to directly cause ALI, we expect that neutrophil depletion will render SS mice resistant to CVF-induced ALI. Consistent with this, we expect that *in vitro* treatment of neutrophils with C5a will induce NET formation. Given the ability of free heme and complement to cause neutrophil injury, we anticipate that CVF and hemin will result in a significant increase in serum markers of endothelial cell activation. We further expect that neutrophil depletion and P-selectin blockade will significantly reduce endothelial injury and subsequent ALI. If no evidence of neutrophil activation or NET formation is observed following CVF or hemin treatment in SS mice, we will measure levels of hemopexin in SS mice to determine if the inability of CVF or hemin treatment to induce NET formation correlates with the ability of mice to scavenge heme. In the event that it is difficult to examine the impact ACP activation has on neutrophil activation and endothelial injury *in vivo*, we will use an endothelialized microfluidic device and intravital microscopy (collaboration discussed with Dr. Wilbur Lam, Emory and Georgia Tech University).

Scientific rigor, robustness and general considerations for Aim 1. We will appropriately blind individuals performing assays and avoid potential gender differences by studying mice from each gender. Statistical consultation and power analysis was completed with a power set to 0.8 and a significance level of 0.05 while choosing mice in each group. To ensure reproducibility, each experiment will be conducted at least 3 times, with at least 5 mice/group.

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Candidate Information:

A. Candidate Background

I have had the unique opportunity of gaining clinical and research experience in India, England and USA. After graduating from medical school in India, I moved to England for pediatric training. During that time, I focused on neonatology, where I performed clinical research that culminated in two oral presentations at the annual meetings of European Society of Pediatric Research, and Royal College of Paediatrics and Child Health with 4 publications. The dynamic nature of erythrocyte physiology in newborns excited me to consider a career in pediatric hematology. I relocated to the USA and started my residency at Michigan State University. During residency, I published two case reports on children with sickle cell disease (SCD) and vascular malformation, and co-authored a book chapter on anemia. During my fellowship training at Cincinnati Children's Hospital Medical Center, my interest in SCD evolved into conceptualizing a research question on the inflammatory pathways involved in its pathophysiology. Under the mentorship of Dr. Theodosia Kalfa, I studied the enzymatic production of reactive oxygen species (ROS) in SCD and explored potential targets to modulate ROS using 'Sickle x NOX1' and 'Sickle x p22Phox' knock out mouse models. This exciting work has been presented at various national and international conferences (Gordon Research Conference 2015 and ASH 2016) and I am currently writing the manuscript for submission in the second quarter of 2018. In addition, I worked on several projects studying the biochemical, rheological and genomic correlation of patients with RBC membrane and enzyme disorders. This has resulted in a total of 6 publications. I also conducted a workshop to accurately diagnose hereditary hemolytic anemia at the annual meeting of American Society of Pediatric Hematology and Oncology in 2016.

Based on an intriguing clinical observation during my hematology training, I have focused my current research on regulation and perturbations in the alternative complement pathway (ACP) in SCD. With current funding as a Sickle Cell Scholar as part of an NIH-UO1 Excellence in Hemoglobinopathies Research Award since late 2016, I have made significant progress in exploring the role of complement in SCD in humans and mice. Currently, 70% of my effort is devoted towards research. I was also awarded a divisional pilot grant to prospectively study ACP activation in children with SCD admitted for vasoocclusive crisis. While my prior bench experience certainly aids in my laboratory skillset and a project poised for success, the Junior Faculty Focused award will immensely help me to gain necessary investigative skills to achieve my goal of becoming an independent investigator.

B. Career Goals and Objectives

My ultimate goal is to become an independently funded RO1 physician scientist with a focus on both translational and basic science approaches to elucidate the role of complement activation in SCD pathophysiology and to develop novel treatment options for them. My short-term goals for this proposal include a) identifying the specific role complement plays in the pathophysiology of acute chest syndrome (ACS), and b) identifying specific biomarkers for ACS that can help in predicting the event and the severity of ACS. While I work towards meeting these research goals, I intend to acquire and improve my laboratory and scientific skills during the JFF award period. My long-term goal is to have developed a strong foundation in basic research principles and to have created a niche in the field of complement biology within the scope of blood disorders.

Despite many advances within the field of SCD over the past decade, there are only 2 FDA approved medications. This could be attributed to the complex pathophysiology of the disease and a lack of strong pharmacological targets for drug discovery. My work thus far has built a foundation for this proposal. I wish to gain more mechanistic understanding between complement pathway, free plasma hemoglobin/heme and organ injury. As my research in complement biology and SCD progressed, I became aware of the need to further enhance my skill sets on laboratory techniques such as confocal microscopy, enzyme-linked immunosorbent assay, *in-vitro* complement assays, neutrophil extracellular trap detection, endothelial perturbation and oxygenation/deoxygenation of sickle red cells. This superior

research model and strong mentorship will vastly help me attain the knowledge, laboratory skills, grantsmanship and help me transition to be in a competitive research environment as well as provide the basis for a competitive application for the NIH K08 Career Development Award in February 2019. In addition, I also plan to simultaneously apply for other career development grants, such as the American Society of Hematology Scholar Award, Doris Duke Charitable Foundation Award and departmental pilot awards.

My clinical effort is 30% with one full day clinic a week and 4 weeks of service a year. In my clinic, I follow a cohort of children with hemolytic and complement disorders, such as RBC disorders, atypical hemolytic uremic syndrome, thrombotic microangiopathy and paroxysmal nocturnal hemoglobinuria. This clinical niche on hemolytic anemia supplements my basic science knowledge, providing me a foresight to guide my research, thereby allowing me to ask further disease-specific research questions. I regularly mentor and teach hematology fellows and 3rd year medical students in their monthly didactic sessions, in addition to an undergraduate, high school student and advanced nurse practitioners.

C. Career Development and Training Activities During Award Period

My advisory committee will include accomplished researchers with track records in mentoring and complementary expertise in fields related to my research. Dr. Sean Stowell (complement biology), Dr. Clinton Joiner (RBC physiology and SCD), and Dr. David Archer (SCD and transgenic mouse models). The JFF award will provide additional funding and protected time as well as facilitate my career development. I aim to achieve this through stable mentorship, ongoing laboratory training, formal course work, participation in professional meetings, grants and manuscript writing and continued clinical responsibilities.

Category	**Activity**	Q 1	Q 2	Q 3	Q 4
Original Research	Specific Aim 1	X	X	X	
Laboratory Training	New laboratory techniques- Stowell lab	X	X		
	Working with mouse models- Archer lab	X	X		
	Confocal microscopy- Stowell lab and Core	X			
	Endothelial perturbation- Stowell/ Archer lab		X	X	
Formal Course Work	Flow Cytometry and Live Imaging Core	X	X		
	Immunology course within graduate program	X	X		
Prof. Meetings	American Society of Hematology		X		
	International Complement Society		X		
Lab Meetings/ Seminars/Mentorship	Weekly Lab Meeting/ Journal Club Weekly meeting with primary mentor Biweekly meeting with co-mentors Quaterly meeting with mentor committee Monthly K-club Seminar	X	X	X	X
	Advances in Research (AiR) Seminar, Aflac	X	X	X	X
Manuscript Prep	First author publications	X			X
Grant Writing	Specific aims revision and refinement		X	X	
	K08 Grant writing			X	X
	Submission of KO8				X