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Augusta, GA 30912

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14. ABSTRACT The overarching theme of our NF YI proposal is to gain mechanistic insight and develop therapeutic targets for the prevention/treatment of neurofibromatosis type 1 (NF1) related cardiovascular disease. Previously, we developed mouse models of arterial stenosis and aneurysm formation that resemble disease in NF1 patients. We have completely interrogated the MCP1/CCR2 signaling pathway in the pathogenesis of NF1 arterial stenosis and a published manuscript is attached to this report. The primary findings of these studies were that CCR2 activation is required for the infiltration of neurofibromin-deficient myeloid cells, the primary cellular effectors of NF1 arterial stenosis, and that pharmacologic inhibition of CCR2 activation is a viable therapeutic option for the prevention and/or treatment of NF1 arterial stenosis. The second aim of our proposal was to interrogate reactive oxygen species production in the generation of NF1 aneurysms. We have initiated work on the proposed experiments using our NF1-related aneurysm model and have generated the mice and tissues for the proposed experiments. We have also begun experiments in a separate model system (calcium chloride) to support our previous findings of enhanced aneurysm formation in Nf1 heterozygous mice.					
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Introduction:

The overarching theme of our NF1YI proposal is to gain mechanistic insight and develop therapeutic targets for the prevention/treatment of neurofibromatosis type 1 (NF1) related cardiovascular diseases. Cardiovascular disease affects upwards of 10% of the more than 2,000,000 persons with NF1 worldwide and presents with lesions in the proximal arteries such as arterial stenosis and aneurysm formation. We have developed murine models that closely resemble NF1 arterial stenosis and aneurysm formation, which are both primarily mediated through the infiltration of bone marrow-derived myeloid cells into the vascular wall in *Nf1* heterozygous mice. However, the pathological consequences of these cells are somewhat opposed, wherein arterial stenosis is the result of smooth muscle cell proliferation and inward remodeling and aneurysms are the result of smooth muscle cell apoptosis and outward remodeling. To better understand how neurofibromin-deficient myeloid cells can lead to different pathological outcomes, we propose to interrogate the recruitment of macrophages via monocyte chemoattractant peptide-1 (MCP-1) stimulation of its receptor (CCR2) and the generation of reactive oxygen species, which are generated in excessive quantities by neurofibromin-deficient macrophages in our arterial stenosis and aneurysm models, respectively.

Keywords:

neurofibromatosis; stenosis; aneurysm; MCP-1; CCR2; reactive oxygen species; superoxide; macrophages; monocytes; arteries; cardiovascular disease

Major Goals and Accomplishments:

Significant progress toward accomplishing the specific aims outlined in our DOD YI proposal has been made over the present reporting period. Outlined below are the original aims and a summary of experimental results and progress to date.

*Aim 1: Test the hypothesis that upregulated MCP-1/CCR2 signaling drives macrophage homing and augments arterial stenosis in *Nf1*^{+/-} mice.*

We have made significant progress in our experimental approach. We have generated the necessary genotypes for all experiments including the compound mutant *Nf1*^{+/-};*CCR2*^{-/-}, *Nf1*^{+/-}, and WT mice. We performed carotid artery ligation in all groups and confirmed our preliminary data and previous observation that *Nf1*^{+/-} mice develop a robust neointima after arterial injury compared to WT mice. Genetic deletion of *CCR2* completely abrogates neointima formation in *Nf1*^{+/-} mice (**Figure 4**, *Hum. Molec. Genet.* 2016). We also showed that *CCR2*-deficient *Nf1*^{+/-} neointimas exhibit a significant reduction in macrophage infiltration after carotid injury as compared to *Nf1*^{+/-} neointimas with intact *CCR2* expression (**Figure 4D**).

Having generated *Nf1*^{+/-};*CCR2*^{-/-} mice required for Aim 1 of the proposal, we have recently completed the generation of chimeric *Nf1*^{+/-} with differential expression of *CCR2* in bone marrow cells. We have initiated experiments to study neointima formation in these mice and anticipate preliminary results from these experiments in the coming months. Additional morphometric and immunohistochemical analysis of *Nf1*^{+/-};*CCR2*^{-/-} and chimeric *Nf1*^{+/-} mice are proceeding as planned and will be completed by the end of FY17.

Cell culture experiments examining the effect of MCP-1 on *Nf1*^{+/-} smooth muscle cell (SMC) function were completed ahead of schedule. To assess *Nf1*^{+/-} SMC proliferation, we isolated SMC from the aortas of *Nf1*^{+/-}, *Nf1*^{+/-};*CCR2*^{-/-}, and WT mice as described. MCP-1 incubation results in a dose-responsive increase in *Nf1*^{+/-} SMC proliferation, which corresponds with activation of the neurofibromin-regulated Erk kinase (**Figure 2A and B**, *Hum. Molec. Genet.* 2016). Genetic deletion of *CCR2* or incubation with a potent Erk inhibitor significantly reduces MCP-1 induced *Nf1*^{+/-} SMC proliferation (**Figure 2A and C**, *Hum. Molec. Genet.* 2016).

In the application, we proposed to study *Nf1*^{+/-} monocyte/macrophage recruitment using a validated peritonitis model. We have initiated experiments in *Nf1*^{+/-}, *Nf1*^{+/-};*CCR2*^{-/-}, and WT mice to

understand the role of CCR2 expression in monocyte/macrophage recruitment using this model. We have used the compound mutant mice for our carotid artery ligation model and have experience limitations in the number of mutant mice available for these experiments. We anticipate having sufficient numbers for data interpretation by the fall of 2016. To provide for mechanistic studies and increase utilization of mice allocated for other experiments, we performed *in vitro* assays of macrophage function in response to MCP-1. Consistent with our preliminary data (**Figure 3**), we show a dose-responsive increase in *Nf1*^{+/-} macrophage proliferation and migration in response to MCP-1 (**Figure 1**, *Hum. Molec. Genet.* 2016). Further, MCP-1 appears to preferentially activate Ras-Akt signaling in *Nf1*^{+/-} macrophages. This is particularly interesting since we previously showed that administration of PD0325901, a potent Erk inhibitor, reduced *Nf1*^{+/-} macrophage recruitment and *Nf1*^{+/-} neointima formation. MCP-1 may function through a distinct mechanism as those studies were performed using LPS. The *in vivo* peritonitis model will provide some insight into this apparent discrepancy.

Major Goals for SA1:

1. Generation of experimental mice (0-12 months)
 - a. We have generated the experimental compound mutant mice outlined in SA1 and have completed the proposed experiments using these mice. A manuscript describing these results was published in *Human Molecular Genetics* in 2016.
2. Generation of chimeric mice (0-12 months)
 - a. In the original application, we also proposed to generate chimera *Nf1*^{+/-} mice via transplantation of WT or *Nf1*^{+/-} bone marrow into *Nf1*^{+/-} recipient mice. We have generated the appropriate experimental and control mice as outlined in the proposal and have initiated experiments in these mice.
3. Carotid artery ligation (6-18 months)
 - a. We have performed carotid artery ligation on our compound mutant mice and have begun to use this technique in chimeric mice generated for this proposal.
4. Analysis of SMC proliferation and migration *in vitro* (0-12 months)
 - a. We have completed experiments in cultured SMC as outlined in SA 1
5. Analysis of macrophage recruitment *in vivo* (6-12 months)
 - a. We initially proposed to study *Nf1*^{+/-} macrophage function *in vivo* using a peritonitis model. To provide more mechanistic insight, we studied cultured *Nf1*^{+/-} and WT macrophage response to MCP-1. Cells were harvested from the bone marrow and derived with macrophage colony stimulating factor (M-CSF). Proliferation and migration along with Ras activity were studied in similar assays proposed in our SMC *in vitro* studies. We showed that MCP-1 is a potent agonist of *Nf1*^{+/-} macrophage proliferation and migration via Erk and Akt activation.
 - b. In order to proceed with experiments using the carotid artery ligation technique and generate sufficient quantities of compound mutant mice for the generation of *Nf1*^{+/-} chimeras, we have experienced a slight delay in initiating experiments to study *in vivo* monocyte/macrophage recruitment. These experiments are currently underway and we anticipate having experimental results in FY17.

Aim 2: Test the hypothesis that enhanced ROS production by Nf1^{+/-} macrophages induces SMC proliferation, thus promoting inward arterial remodeling.

We have initiated breeding strategies to generate *Nf1*^{+/-};*p47*^{-/-} mice as outlined in the Statement of

Work. In the coming fiscal year, we anticipate implanting AngII osmotic pumps in our first cohort in early 2017 with preliminary results expected by Spring 2017.

Major Goals for SA2:

1. Generation of *Nf1*^{+/-};*p47*^{phox}^{-/-} mice (12-36 months)
 - a. We have initiated breeding strategies to generate these compound mutant mice and anticipate experiments related to SA2 to commence in early 2017
2. Angiotensin II infusion
 - a. We anticipate implanting osmotic pumps in our first cohort in early 2017. This timeline impacts Major Goals 2C, 2D, and 2E. We anticipate work on these Major Goals to follow the anticipated timeline in the Statement of Work.

Opportunities for training and professional development

Nothing to report

Dissemination of Results

Results from SA1 were recently presented at the Southern Society for Pediatric Research and the Children's Tumor Foundation Annual Meeting. Our work was recognized as **Basic Science Young Investigator Award** at the SSPR meeting and **Best Poster Award** at the annual CTF meeting.

Plan for reporting in coming fiscal year

Nothing to report

Impact:

Our experimental results from SA1 (published in *Human Molecular Genetics*) are critical to forming a comprehensive understanding of arterial stenosis in persons with NF1. We provide preclinical evidence that CCR2 expression is critical for *Nf1*^{+/-} neointima formation and deletion of *CCR2* limits neointima formation and arterial stenosis in *Nf1*^{+/-} mice. Further, our experimental results are likely to have broader applications to other manifestations of NF1 since MCP-1 is a potent agonist of other myeloid-derived cell lines, which have been implicated in myeloid-specific leukemias and solid tumors.

Technology impact

Nothing to report

Impact on Society

Nothing to report

Changes to Report:

Nothing to report

Products:

Published Manuscripts (*denotes federal support)

1. *Bessler WK, Kim G, Hudson FZ, Mund JA, Mali R, Menon K, Kapur R, Clapp DW, Ingram DA, **Stansfield BK**. "Nf1+/- Monocytes/Macrophages Induced Neointima Formation via CCR2 Activation". *Human Molecular Genetics*. 2016 Mar 1;25(6):1129-1139.

Abstracts

1. ***Stansfield BK**, Ingram DA. *CCR2 Signaling is Necessary for Nf1^{+/-} Neointima Formation*, Southern Society for Pediatric Research, New Orleans, LA
2. Benson TW, Chatterjee TK, Weintraub DS, Popoola O, **Stansfield BK**, Crowe M, Pillai A, Mintz J, Stepp D, Brittain J, Bogdanov V, Weintraub NL. *Duffy Antigen Receptor for Chemokines Modulates Adipose Inflammation in Obesity Related Metabolic Disease*, American Diabetes Association, Boston, MA
3. *Bessler,WK, Hudson FZ, Fulton DJ, Ingram DA, **Stansfield BK***. *Neurofibromin Regulates Oxidative Stress and Arterial Remodeling*, Children’s Tumor Foundation, Monterey, CA ***Award for Basic Science Poster**
4. Benson TW*, Chatterjee TK, Weintraub DS, Popoola O, Joseph J, **Stansfield BK**, Crowe M, Yiew N, Unruh D, Pillai A, Williams j, Mintz J, Stepp D, Brittain J, Bogdanov V, Weintraub NL. *The Role of the Duffy Antigen Receptor for Chemokines in Metabolic Disease*, Experimental Biology, San Diego, CA

Participants:

Name:	Brian Stansfield, MD
Project Role:	Principal Investigator
Person Months:	6
Contribution:	Carried out experiments, results interpretation, data management
Funding Source:	W81XWH-15-1-022

Name:	Farlyn Hudson
Project Role:	Research Associate
Person Months:	6
Contribution:	Carried out experiments, colony management, breeding
Funding Source:	W81XWH-15-1-022

Funding changes for PI or key personnel:

PI: no changes in funding

Collaborators/Consultants

Neal Weintraub

1R01HL126949-01A1 03/01/2016 – 02/29/2020 2.4 Calendar Months
NIH/NHLBI
“Epigenetic regulation of HDAC9 in obesity and atherosclerosis”
Role: Principal Investigator

1R01AR070029-01 04/01/2016 – 03/31/2021 0.6 Calendar Months
NIH/NIAMS
“Innovative Approaches to Treat Duchenne Muscular Dystrophy Using iPSC-Derived Muscle Progenitors”
Role: Principal Investigator of MPI grant

2R01HL086555-08A1 03/01/2008 – 03/31/2020 1.2 Calendar Months
NIH/NHLBI
“Hypoxia and cardiac stem cell homing”

Role: Co-Investigator

David Fulton

1R01HL124773-02 04/01/2015 – 03/31/2019 NIH/ 2.4 Calendar Months
NHLBI

Title: “Novel mechanistic pathways of cardiovascular disease in obesity” Role: Principal Investigator

1-16-IBS-196 01/01/2016 – 12/31/2018 0.36 Calendar Months
American Diabetes Association

Title: “Increased pneumonia-associated pulmonary barrier dysfunction in type 2 diabetes”

Role: Co-Investigator

1R01HL125926-01A1 02/01/2016 – 01/31/2020 2.4 Calendar Months
NIH/NHLBI

Title: “Galectin-3: A mediator of vascular remodeling in pulmonary arterial hypertension”

Role: Contact Principal Investigator

Other organizations involved in the project:

None to report

Special reporting:

None to report