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14. ABSTRACT

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 chemotactic 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.

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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 chemotactic 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 completed the proposed experiments in Aim 1. We reported in a previous publication that compound mutant *Nf1+/-;CCR2-/-* do not develop neointima formation and arterial remodeling closely resembles our observations in WT C57Bl/6 mice. This is in stark contrast to *Nf1*+/- mice with intact CCR2 expression, which develop a robust neointima with marked macrophage infiltration. We also showed that treatment of *Nf1*+/- mice with a potent CCR2 inhibitor following carotid artery ligation rescues the phenotype and may be a viable therapeutic option for NF1 patients with arterial stenosis.

We have also completed the bone marrow transplant studies, which introduce *CCR2* knockout marrow into *Nf1*+/- animals. Following engraftment, *Nf1*+/- mice with CCR2-deficient and intact bone marrow cells were subjected to carotid artery ligation and neointima formation was studied as described. We showed that CCR2 signaling in bone marrow cells is required to produce the enhanced neointima observed in *Nf1*+/- mice. Collectively, these data strongly implicate that CCR2 expression is required for NF1-arterial stenosis.

As mentioned in the previous report, we have completed *in vitro* studies to assess MCP-1 induced *Nf1*+/- SMC proliferation and migration. The results of these experiments were published in *Hum Molec. Genet.* in 2016. We observed that MCP-1 appears to preferentially activate Ras-Akt signaling in *Nf1*+/- macrophages, which is surprising considering our previous observation that Mek-Erk inhibition showed a dose-responsive reduction in *Nf1*+/- neointima formation (American Journal of Pathology, 2014). In follow up experiments, we have identified that inflammatory *Nf1* knockout macrophages display enhanced Akt activation; whereas, patrolling macrophages demonstrate more Erk activation. MCP-1, largely thought to mobilize inflammatory monocytes, may enhance M1 (inflammatory) macrophage mobilization. We are currently working extensively to understand differential activation of Ras kinases in these macrophage subpoulations.

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. We have generated the appropriate experimental and control mice as outlined in the proposal and have initiated experiments in these mice. Please see **Figure 1 and Figure 2** in the appendix for experimental results.

3. Carotid artery ligation (6-18 months)
    a. We have performed carotid artery ligation on our compound mutant mice and 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. Please see **Figure 3** in the appendix for results of *in vivo* macrophage recruitment in *Nf1*+/- and WT mice.

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

We have generated the first cohorts of *Nf1*+/-; *p47*-/- mice for use in our proposed experiments. Generating sufficient male mice for these experiments has been slower than expected, coupled with our use of these mice in our carotid artery ligation model has slowed our progress toward completing SA2. To date, we have completed two cohorts of animals and are presently analyzing aneurysm formation in the second cohort. We anticipating the need for large quantities of these mice and proposed a breeding timeline of 12-36 months in our proposal. As male mice come available and having completed other work using these mice, we anticipate making significant progress toward the completion of SA2 in FY18.

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. As described, generating sufficient quantities of matched male mice has been slow and we have progressed through two cohorts to date.

2. Angiotensin II infusion
    a. We have implanted osmotic pumps in two cohorts of mice to date. At this time, we are analyzing the second cohort and are unable to draw any conclusions at present. We have attached images of aneurysms isolated from the first cohort in **Figure 4** in the appendix. This timeline impacts Major Goals 2C, 2D, and 2E. As we had an extended timeline for generating these mice in the Statement of Work, our progress to date falls within our original estimate, but we have experienced technical challenges in generating these mice.

3. Analysis of *Nf1*+/- SMC apoptosis
    a. We have initiated experiments in *Nf1*+/- and WT smooth muscle cells (SMC) to identify discrete molecular pathways involved in *Nf1*+/- SMC apoptosis. Interestingly, we found that Erk activity is markedly increased in *Nf1*+/- SMC in response to AngII and that hydrogen peroxide enhances *Nf1*+/- SMC apoptosis. Presently, we are generating *Nf1*+/- and WT macrophages for co-culture with *Nf1*+/- and WT SMC to identify how *Nf1*+/- macrophages initiate SMC apoptosis in the pathogenesis of aortic aneurysm formation. Please see **Figure 5** in the appendix for these results.

Opportunities for training and professional development

Nothing to report

Dissemination of Results

Results from SA1 were published in *Human Molecular Genetics* in 2016

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. Complimentary to these results is our findings that *Nf1*+/- macrophage generated superoxide is directly involved in neointima formation provides a rationale target for both NfF1-related arterial stenosis and aneurysm formation. In broader context, a greater understanding of how neurofibromin controls macrophage function directly informs other manifestations of Nf1 for which macrophages participate in disease initiation or progression including myeloid leukemias, neurofibromas, and MPNST.

Technology impact

Nothing to report

Impact on Society

Nothing to report

Changes to Report:

Nothing to report

Products:

Published Manuscripts (*denotes federal support)

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5. Kim HW, Blomkalns AL, Ogbi M, Thomas M, Gavrila D, Neltner BS, Cassis LA, Thompson RW, Weiss RW, Lindower PD, Blanco VM, McCormick ML, Daugherty A,Fu X, Hazen SL, **Stansfield BK**, Huo Y, Chatterjee T, Weintraub NL. Role of myeloperoxidase in abdominal aortic aneurysm formation: mitigation by taurine. Am J Physiol - Heart Circ Physiol. *In Press*
6. Benson TW, Chatterjee TK, Weintraub DS, Popoola O, Joseph J, **Stansfield BK**, Crowe M, Yiew KH, Unruh D, Pillai A, Williams J, Mintz J, Stepp DW, Brittain J, Bogdanov V, Weintraub NL. "Duffy Antigen Receptor for Chemokines Regulates Insulin Signaling and Adipocyte Maturation". *Submitted*.
7. *Zhang H, Hudson FZ, Xu Z, Tritz R, Rojas M, Patel C, Haigh SB, Bordan Z, Ingram DA, Fulton DJ, Weintraub NL, Caldwell RB, **Stansfield BK.** "Neurofibromin Deficiency Induces Endothelial Cell Proliferation and Retinal Neovascularization." *Invest Ophthalmol Vis Sc*. Submitted

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
5. Tritz R, Zhang H, Fulton DJ, **Stansfield BK**. *Metabolic Characterization of Circulating Human Endothelial Colony Forming Cells*. Southern Society for Pediatric Research, New Orleans, LA
6. *Tritz R, Zhang HB, Hudson FZ, Benson TW, Kim HW, Fulton DJ, Weintraub NL, **Stansfield BK.** *Neurofibromin is a Novel Regulator of Macrophage Polarization via PFKFB3 Activation.* Children's Tumor Foundation, Washington D.C.

Participants:

Name:	Brian Stansfield, MD
Project Role:	Principal Investigator
Person Months:	1.8
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

Name:	Val Harris
Project Role:	Research Associate
Person Months:	4.8
Contribution:	Carried out experiments, animal surgeries
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Collaborators/Consultants

Neal Weintraub

4R01HL112640-05 07/13/2012 – 11/30/2017 (NCE) 2.4 Calendar Months
NIH/NHLBI $272,379 Annual Direct Costs
"Perivascular adipose tissue and vascular remodeling"
Role: Principal Investigator

5R01HL126949-02 03/01/2016 – 02/29/2020 2.4 Calendar Months
NIH/NHLBI $295,000 Annual Direct Costs
"Epigenetic regulation of HDAC9 in obesity and atherosclerosis"
Role: Principal Investigator

5R01AR070029-02 04/01/2016 – 03/31/2021 0.6 Calendar Months
NIH/NIAMS $427,048 Annual Direct Costs
"Innovative Approaches to Treat Duchenne Muscular Dystrophy Using iPSC-Derived Muscle Progenitors"
Role: Principal Investigator of MPI grant

5R01HL086555-09 05/01/2016 – 03/31/2020 1.2 Calendar Months
NIH/NHLBI $250,000 Annual Direct Costs
"Hypoxia and cardiac stem cell homing"
Role: Co-Investigator

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NIH/NHLBI $485,000 Annual Direct Costs
"Notch1/miR-322 axis in stem cell mediated vascular repair"
Role: Principal Investigator of MPI grant

David Fulton

5R01HL124773-03 04/01/2015 – 02/28/2019 2.4 Calendar Months
NIH/NHLBI $175,669 Annual Direct Costs
"Novel mechanistic pathways of cardiovascular disease in obesity"
Role: Principal Investigator of MPI grant

1-16-IBS-196 (Lucas – PI) 01/01/2016 – 12/31/2018 0.36 Calendar Months
American Diabetes Association $104,545 Annual Direct Costs
"Increased pneumonia-associated pulmonary barrier dysfunction in type 2 diabetes"
Role: Co-Investigator

5R01HL125926-02 02/01/2016 – 01/31/2020 2.4 Calendar Months
NIH/NHLBI $176,975 Annual Direct Costs
"Galectin-3: A mediator of vascular remodeling in pulmonary arterial hypertension"
Role: Contact Principal Investigator of MPI grant

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Original article

Neurofibromin is a novel regulator of Ras-induced reactive oxygen species production in mice and humans

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ABSTRACT

Neurofibromatosis type 1 (NF1) predisposes individuals to early and debilitating cardiovascular disease. Loss of function mutations in the *NF1* tumor suppressor gene, which encodes the protein neurofibromin, leads to accelerated $p21^{Ras}$ activity and phosphorylation of multiple downstream kinases, including Erk and Akt. *Nf1* heterozygous ($Nf1^{+/-}$) mice develop a robust neointima that mimics human disease. Monocytes/macrophages play a central role in NF1 arterial stenosis as *Nf1* mutations in myeloid cells alone are sufficient to reproduce the enhanced neointima observed in $Nf1^{+/-}$ mice. Though the molecular mechanisms underlying NF1 arterial stenosis remain elusive, macrophages are important producers of reactive oxygen species (ROS) and Ras activity directly regulates ROS production. Here, we use compound mutant and lineage-restricted mice to demonstrate that $Nf1^{+/-}$ macrophages produce excessive ROS, which enhance $Nf1^{+/-}$ smooth muscle cell proliferation *in vitro* and *in vivo*. Further, use of a specific NADPH oxidase-2 inhibitor to limit ROS production prevents neointima formation in $Nf1^{+/-}$ mice. Finally, mononuclear cells from asymptomatic NF1 patients have increased oxidative DNA damage, an indicator of chronic exposure to oxidative stress. These data provide genetic and pharmacologic evidence that excessive exposure to oxidant species underlie NF1 arterial stenosis and provide a platform for designing novels therapies and interventions.

1. Introduction

Neurofibromatosis type 1 (NF1) is a common genetic disorder resulting from germline mutations in the *NF1* tumor suppressor gene and affects over 2 million people worldwide [1]. Neurofibromin, the protein product of *NF1*, functions as a catalyst for the slow, intrinsic hydrolysis of active $p21^{Ras}$ (Ras) [1]. Thus, loss of neurofibromin expression increases Ras-dependent kinase activity in response to growth factor stimulation of receptor tyrosine kinases. The downstream Ras kinases Erk and Akt turn on multiple molecular switches to promote a pro-survival phenotype in neurofibromin-deficient cells.

Persons with NF1 have a strong predisposition for cardiovascular disease, which often presents in adolescence and early adulthood [2–4]. Upwards of 8% of NF1 patients will develop hypertension, arterial stenosis, aortic aneurysms, or moyamoya,

Abbreviations: EEL, external elastic lamina; IEL, internal elastic lamina; I/M ratio, intima/media ratio; MNC, peripheral blood mononuclear cells; NF1, neurofibromatosis type 1; $Nf1^{+/-}$, heterozygous for the *Nf1* allele; $Nf1^{+/-}$;$p47^{-/-}$, heterozygous for the *Nf1* allele and homozygous deletion of $p47^{phox}$; $Nf1^{flox/+}$;$LysM^{cre}$, heterozygous for the *Nf1* allele in myeloid cells alone; $Nf1^{flox/flox}$;$LysM^{cre}$, homozygous for the *Nf1* allele in myeloid cells alone; $Nf^{flox/+}$;$gp91^{flox/flox}$;$LysM^{cre}$, heterozygous for the *Nf1* allele and homozygous deletion of $gp91^{phox}$ in myeloid cells alone; NOX2, NADPH oxidase 2; PMA, phorbol myristate acid; Ras, $p21^{Ras}$ pathway; ROS, reactive oxygen species; SMC, smooth muscle cell; SOD, superoxide dismutase; WT, wild type

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though universal screening has not been adopted and may underestimate the true prevalence of disease [4–6]. The distribution of NF1 vasculopathy within a single patient is often patchy and affects multiple vessels [4]. The varied presentation of arterial lesions suggests that NF1 patients may require a "second hit" mutation in the normal *NF1* allele or, more likely, a local insult in the vessel wall leading to dysregulation of normal repair mechanisms. Constitutional homozygosity for *NF1* mutations is embryonic lethal in humans and mice; therefore, inherited mutations in a single *NF1* allele are likely sufficient for the increased disease prevalence and provide a platform for investigation [7].

We have developed a mouse model of NF1 arterial stenosis using *Nf1* heterozygous ($Nf1^{+/-}$) mice that phenotypically resembles human NF1 arterial lesions [8–12]. Following carotid artery injury, $Nf1^{+/-}$ mice develop a robust neointima when compared with WT mice, which is characterized by α-SMA positive smooth muscle cells (SMC) and a predominance of bone marrow-derived macrophages within the neointima [10–12]. Disruption of PDGF-Ras-Erk signaling inhibits $Nf1^{+/-}$ SMC proliferation and prevents neointima formation in $Nf1^{+/-}$ mice[9]; however, SMC-specific *Nf1* heterozygosity failed to replicate the enhanced neointima observed in $Nf1^{+/-}$ mice and provides evidence that other cell populations are required to initiate neointima formation in $Nf1^{+/-}$ mice [8,10]. In support of this hypothesis, WT mice reconstituted with $Nf1^{+/-}$ bone marrow developed a pronounced neointima following carotid artery ligation while $Nf1^{+/-}$ mice reconstituted with WT bone marrow developed a modest neointima similar in size to WT lesions [8]. Further, we recently showed that loss of a single *Nf1* gene copy in myeloid cells is sufficient to reproduce the exaggerated arterial lesions observed in $Nf1^{+/-}$ mice [11]. These experiments in lineage-restricted and chimeric mice provide strong evidence that neurofibromin-deficient monocytes and macrophages are critical mediators of $Nf1^{+/-}$ arterial stenosis. However, the mechanisms through which $Nf1^{+/-}$ monocytes and macrophages directly influence $Nf1^{+/-}$ SMC proliferation and arterial stenosis is completely unknown.

Emerging evidence suggests that Ras kinases directly regulate reactive oxygen species (ROS) production and, in turn, ROS may modulate Ras activity [13–15]. Constitutive activation of Ras in hematopoietic progenitor and cancer cells dramatically increases ROS production via activation of the NADPH oxidase complex [16–19]. Also, *Drosophila* harboring mutations in the *Nf1* gene exhibited shortened lifespan and increased production of and vulnerability to ROS [20,21], while overexpression of neurofibromin prolonged lifespan and reduced ROS production [22]. More recently, neurofibromin deficiency or Ras activation significantly increased oligodendrocyte ROS production and disrupted endothelial tight junctions, which was restored by daily administration of the antioxidant N-acetyl cysteine [23]. These findings are intriguing since neurofibromin occupies a unique position in the regulation of kinases that activate ROS production and enhance SMC proliferation [24–26]. For example, the Ras-dependent kinases Akt and Erk directly phosphorylate the $p47^{phox}$ subunit of NADPH oxidase 2 (NOX2) and facilitate Rac2-dependent recruitment of $p67^{phox}$ to the transmembrane component of NOX2 to increase superoxide production in phagocytes [27–29]. Overproduction of ROS in infiltrating leukocytes via NOX2 therefore may augment SMC function and participate in the pathogenesis of arterial lesions in NF1 patients. Therefore, we hypothesize that loss of neurofibromin in monocytes/macrophages enhances ROS production via NOX2 activation and amplifies $Nf1^{+/-}$ SMC proliferation leading to occlusive arterial disease. As a corollary to our experimental murine work, we seek to identify whether NF1 patients experience chronic oxidative stress.

2. Materials and methods

2.1. Animals

Protocols were approved by Laboratory Animal Services at Augusta University and Indiana University. $Nf1^{+/-}$ mice were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA) and backcrossed 13 generations into the C57BL/6J strain. $p47^{phox}$ (4742) knockout mice ($p47^{-/-}$) were purchased from The Jackson Laboratory and maintained on C57BL/6 strain. $Nf1^{+/-}$ mice were intercrossed with $p47^{-/-}$ mice to produce $Nf1^{+/-}$;$p47^{-/-}$ mice. $Nf1^{flox/flox}$ mice were obtained from Luis Parada (University of Texas Southwestern Medical Center, Dallas, TX) and maintained on C57BL/6 background. $gp91^{flox/flox}$ mice were obtained from Abay Shah (King's College, London, UK). *LysMcre* (4781) mice were purchased from The Jackson Laboratory and maintained on C57BL/6 background. $Nf1^{flox/flox}$ mice were crossed with $gp91^{flox/flox}$ and *LysMcre* mice to generate $Nf^{flox/+}$;$gp91^{flox/flox}$;*LysMcre* mice (heterozygous loss of *Nf1* and homozygous loss of $gp91^{phox}$ in myeloid cells only). LysM is expressed in neutrophils and macrophages. *Cre*-mediated recombination was confirmed by PCR as previously described [11]. Inbreeding of $Nf1^{flox/flox}$ mice with *LysMcre* mice yielded $Nf1^{flox/+}$;*LysMcre* (heterozygous loss of *Nf1* in myeloid cells alone) and $Nf1^{flox/+}$ (WT) controls. Male mice, between 12 and 15 weeks of age, were used for experiments.

2.2. Carotid artery ligation

Carotid artery injury was induced by ligation of the right common carotid artery as previously described [11]. Briefly, mice were anesthetized by inhalation of an isoflurane (2%)/oxygen (98%) mixture. Under a dissecting scope, the right carotid artery was exposed through a midline neck incision and ligated proximal to the bifurcation using a 6-0 silk suture. The contralateral carotid artery was sham ligated as a control. Mice were administered 15 μg of buprenorphine (IP) following the procedure and recovered for 28 days. Whole ligated and control arteries were harvested from experimental mice for analysis as previously described.

2.3. Morphometric analysis

Van Gieson-stained arterial cross Sections 400, 800, and 1200 μm proximal to the ligation were analyzed for neointima formation using Image J (NIH, Bethesda, MD). Lumen area, area inside the internal elastic lamina (IEL), and area inside the external elastic lamina (EEL) were measured for each cross section. To account for potential thrombus formation, arteries containing significant thrombus (> 50% lumen occlusion) at 400 μm proximal to the ligation were excluded from analysis. The number of excluded arteries was not different between experimental groups. Representative photomicrographs for each figure are taken from arterial cross sections between 600 and 1200 μm proximal to the bifurcation. Intima area was calculated by subtracting the lumen area from the IEL area, and the media area was calculated by subtracting the IEL area from the EEL area. Intima/media (I/M) ratio was calculated as intima area divided by media area.

2.4. Arterial ROS detection in vivo

Carotid arteries from $Nf1^{+/-}$ and WT mice were injured as described above. Forty-eight hours after injury, dihydroethidium (20 mg/kg) was provided via IP injection. After an additional 24-h recovery period, mice were sacrificed and whole control and injured carotid arteries were perfused with heparinized saline and flash frozen in OCT compound. Arterial cross sections (20 μm)

were analyzed for fluorescence using an EVOS FL microscope. In a second set of experiments, whole arteries from *Nf1*$^{+/-}$ and WT mice were harvested 72 h after injury and pooled, minced into a single cell suspension, and placed in boiling sodium dodecyl sulfate (SDS) buffer. NOX2 and β-actin expression were analyzed by western blot.

2.5. Isolation of bone marrow-derived macrophages, characterization, and ROS determination

Bone marrow-derived macrophage isolation and characterization was performed as described [8]. To assess ROS production, WT, *Nf1*$^{flox/+}$*;LysMcre*, and *Nf1*$^{flox/flox}$*;LysMcre* macrophages (5×10^5 cells) were suspended in 0.5 mL Hank's Balanced Salt Solution (HBSSS) and stimulated with 100 nM phorbol myristate acid (PMA). L-012 (2 μM) with and without 50 units/mL superoxide dismutase (SOD) was added to the suspension and luminescence was measured at 15-s intervals in a luminometer. In some experiments, macrophages were incubated with PD0325901 (10 nM) or wortmannin (50 nM) to specifically inhibit Erk and Akt activity, respectively. All experiments were performed in triplicate in four distinct cohorts.

2.6. Smooth muscle cell isolation and proliferation

Smooth muscle cell isolation and proliferation assays were performed as described [26]. SMC were obtained by outgrowth from explants of WT and *Nf1*$^{+/-}$ thoracic aortas. SMC were cultured in DMEM supplemented with 20% fetal bovine serum and 100 U/ml penicillin/streptomycin in a 37 °C, 5% CO_2-humidified incubator. For cell proliferation, SMC (5000 cells/cm^2) were placed in a 96-well plate and deprived of growth factors for 12–18 h. Quiescent SMC were stimulated with H_2O_2 (1 and 100 μM) for 24 h and pulse-labeled with 1 μCi/ml of [^{3}H] thymidine for 6 h. β emission was measured and reported as counts per minute. Cell counts using a hemocytometer were performed to confirm radioisotope results. Under the same conditions, cell viability was assessed by MTT assay and light absorbance was measured using a plate reader (570 nm). All experiments were performed in triplicate in four distinct cohorts.

2.7. Patient recruitment

NF1 patients were recruited by the Indiana University NF1 Clinic at Riley Hospital for Children. All patients received a physical examination and a medical history was taken to confirm the diagnosis of NF1 according to the NIH clinical criteria [30]. Patients with a history of cancer, on anti-cancer drugs or pregnant were excluded from the study. All patients gave informed consent prior to participation in the study.

2.8. Isolation of human peripheral blood mononuclear cells and analysis

Blood samples were collected from NF1 patients (37.6 ± 9.7 years) and age- and sex-matched healthy controls (40.2 ± 8.1 years) into EDTA Vacutainer tubes (BD Biosciences). Peripheral blood mononuclear cells (MNCs) were isolated from 16 ml of peripheral blood by density centrifugation using Ficoll-Paque Plus (GE Healthcare) as previously described [31]. A total of 1×10^6 MNCs were resuspended in PBS with 2% FBS and incubated with human FcR Blocking Reagent (Miltenyi Biotec) for 10 min at 4 °C. After blocking, MNCs were incubated for 30 min at 4 °C with the following primary conjugated monoclonal antibodies: anti–human CD14–PECy5.5 (Abcam), anti–human CD45–allophycocyanin–Alexa Fluor 750 (Invitrogen), and anti-human CD16–PECy7 (BD Biosciences — Pharmingen), as well as the live/dead marker ViVid (Invitrogen). After staining, MNCs were washed 2 times with PBS with 2% FBS and fixed in 1% formaldehyde (Sigma-Aldrich) for a minimum of 24 h. Stained MNC samples were acquired on a BD LSRII flow cytometer equipped with a 405-nm violet laser, 488-nm blue laser, and 633-nm red laser. At least 300,000 events were collected for each sample. Data were collected uncompensated and analyzed using FlowJo software version 8.7.3 (Tree Star).

2.9. Modified comet assay in human mononuclear cells

MNCs were assayed for oxidative DNA damage as previously described, with modification [32]. Following cell lysis, slides were treated with formamidopyrimidine DNA glycosylase (FPG), which recognized and removed oxidized purines, causing a DNA break. For each sample, 100 cells were analyzed. The Institutional Review Board of the Indiana University School of Medicine approved all protocols using human tissue samples.

2.10. Statistics

All values are presented as mean ± S.E.M. unless otherwise noted. Human monocyte frequency and leukocyte oxidative-DNA damage was analyzed using Student's *t*-test and sample distribution was analyzed by *F*-test. Macrophage ROS production and SMC proliferation and viability was analyzed using 2-way ANOVA with Tukey's post-hoc test for multiple comparisons. Intima area and I/M ratio analysis was assessed by 1-way ANOVA with Tukey's post-hoc test for multiple comparisons. Murine experiments using apocynin treatment were assessed using 2-way ANOVA with Tukey's post-hoc test for multiple comparisons. Analysis was performed using GraphPad Prism version 5.0 d. $P < 0.05$ were considered significant.

3. Results

3.1. Neurofibromin-deficient macrophages produce excessive superoxide via p21^{Ras} activation

The p21^{Ras} pathway directly, but not completely, regulates intracellular and extracellular oxidant species concentration; therefore, Ras activation in neurofibromin-deficient leukocytes may increase ROS production. To examine the role of neurofibromin-deficiency in leukocyte-mediated ROS production, we utilized cre/lox technology to isolate *Nf1* heterozygous and homozygous macrophages. As *Nf1* nulzygosity results in embryonic lethality, we isolated bone marrow macrophages from *Nf1*$^{flox/+}$*;LysM*^{cre} (*Nf1* heterozygous in myeloid cells) and *Nf1*$^{flox/flox}$*;LysM*^{cre} (*Nf1* homozygous in myeloid cells) and subjected them to stimulation with phorbol myristate acetate (PMA) to provoke ROS production. In response to PMA stimulation, neurofibromin-deficient macrophages generated greater quantities of ROS when compared to WT macrophages (Fig. 1). In fact, a gene-dosage response was observed in neurofibromin-deficient macrophages (Fig. 1A and B). The addition of superoxide dismutase (SOD) effectively quenched ROS production, indicating that *Nf1* mutant macrophages produce excessive superoxide primarily (Fig. 1B).

To examine whether Ras activation in neurofibromin-deficient macrophages directly regulates ROS production, we elicited ROS production in WT and *Nf1*$^{+/-}$ macrophages with PMA in the presence of PD0325901, an inhibitor of Ras-Mek-Erk, and wortmannin, an inhibitor of Ras-PI-3K. In response to PMA, *Nf1*$^{+/-}$ macrophages produced significantly more ROS when compared with WT macrophages and this response was completely inhibited in the presence of either Erk or Akt inhibitors (Fig. 1C). WT macrophages

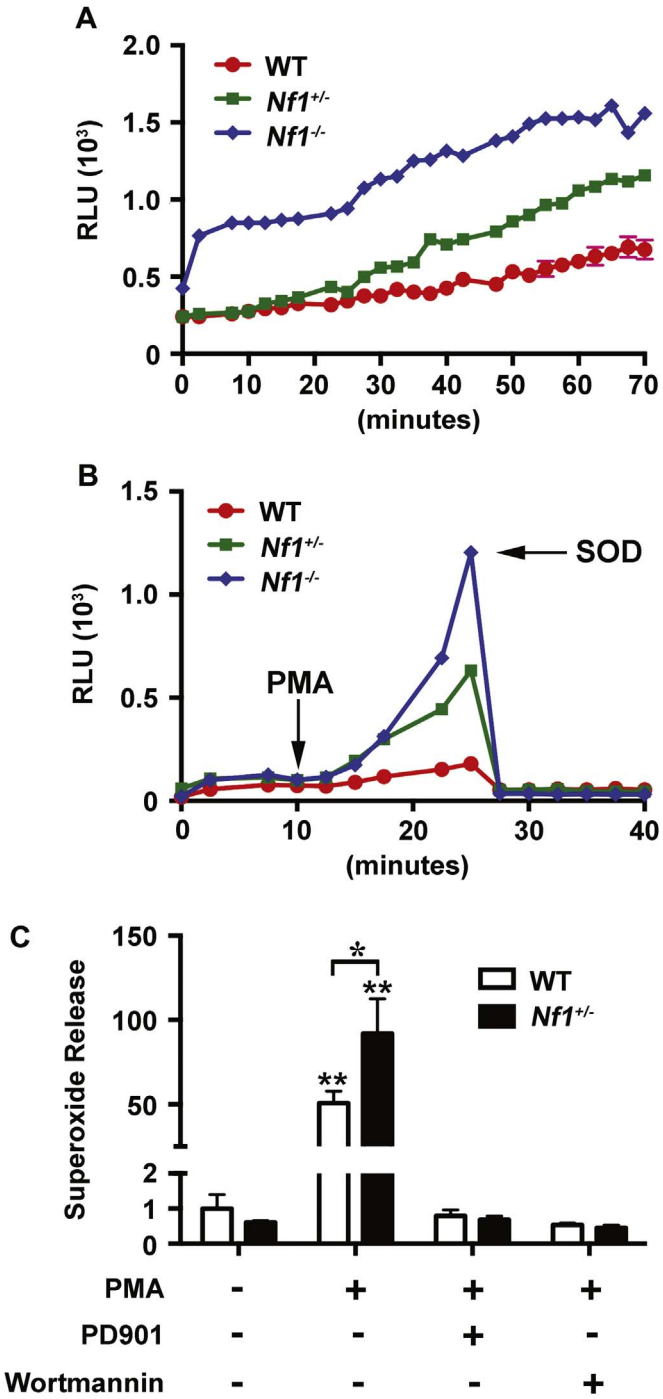

Fig. 1. Neurofibromin regulates ROS production via $p21^{Ras}$. WT (red), $Nf1^{+/-}$ (green), and $Nf1^{-/-}$ (blue) macrophage ROS production in response to phorbol myristate acid (PMA) in the presence or absence of $p21^{Ras}$ inhibitors. A and B. Data represent relative light units in response to PMA (A) and in response to PMA with the addition of superoxide dismutase (B) at indicated time point. C. Data represent fold change ± S.E.M. (n=4) for WT (white bars) and $Nf1^{+/-}$ (black bars) macrophage maximal superoxide production in response to PMA (30 min) in the presence or absence of PD0325901 (10 nM) and wortmannin (50 nM). All comparisons are referred are in reference to unstimulated WT macrophages. $^{*}P<0.01$ for WT versus $Nf1^{+/-}$ macrophages stimulated with PMA. $^{**}P<0.001$ for WT and $Nf1^{+/-}$ macrophages stimulated with PMA versus WT and $Nf1^{+/-}$ macrophages stimulated with PMA in the presence of either PD0325901 or wortmannin.

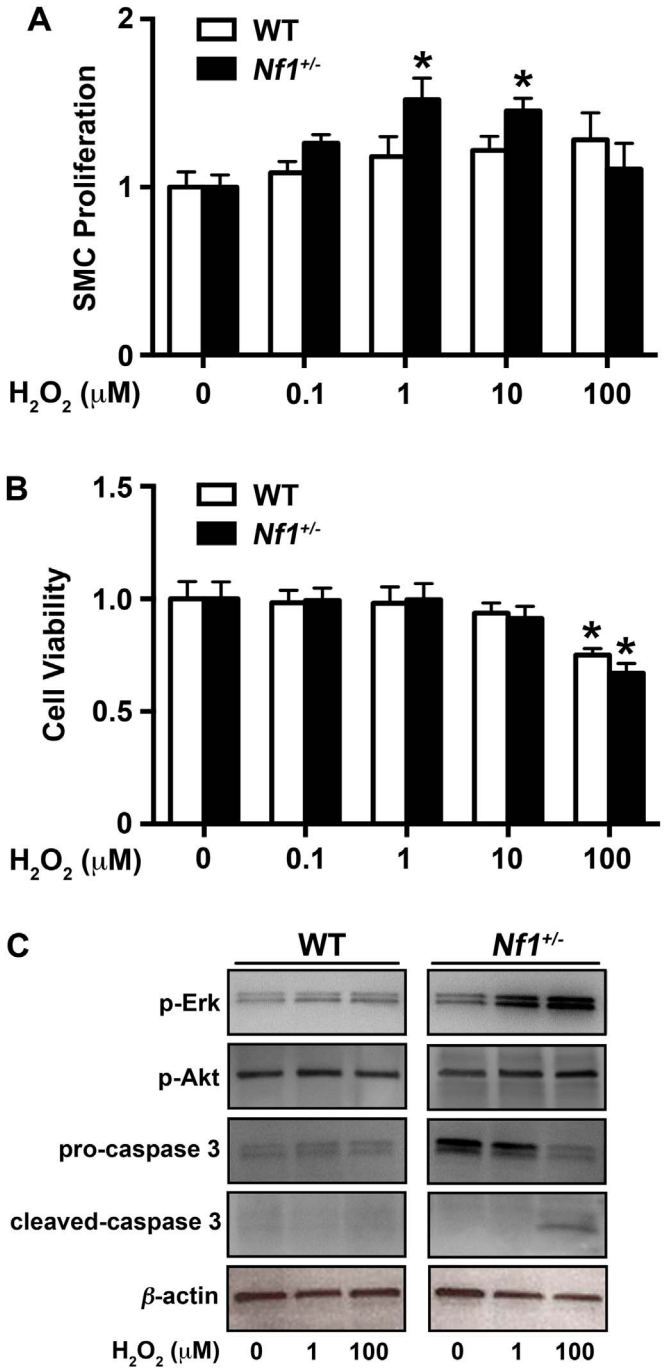

Fig. 2. Low dose H_2O_2 induces $Nf1^{+/-}$ SMC proliferation and Erk activation. WT (white bars) and $Nf1^{+/-}$ (black bars) SMC proliferation in response to stimulation with indicated concentration of H_2O_2 (μM). A. Data represent genotype-specific fold change ± SEM, n=4. $^{*}P<0.05$ for $Nf1^{+/-}$ SMC versus $Nf1^{+/-}$ SMC stimulated with indicated concentration of H_2O_2. B. Data represent genotype-specific SMC viability ± SEM (n=4) in the presence of H_2O_2. $^{*}P<0.01$ for $Nf1^{+/-}$ SMC versus $Nf1^{+/-}$ SMC stimulated with indicated concentration of H_2O_2. C. Representative western blots of phospho-Erk, phospho-Akt, pro-caspase 3, cleaved caspase 3, and β-actin in WT and $Nf1^{+/-}$ SMC treated with indicated concentration of H_2O_2 (n=4).

also produced little ROS when co-incubated with PD0325901 or wortmannin, which suggests that PMA likely induces ROS production via Ras activation.

3.2. $Nf1^{+/-}$ SMC are sensitive to oxidative stress

Reactive oxygen species modify the response of intracellular signaling pathways, including $p21^{Ras}$, and may exaggerate the response of extracellular growth signals to induce cell proliferation and survival. More specifically, superoxide and its primary by-product hydrogen peroxide (H_2O_2) are important mitogens for SMC [33–36]. Based on the observation that neurofibromin-

deficient macrophages produce excessive ROS, we isolated SMC from the aortas of WT and *Nf1*$^{+/-}$ mice to interrogate the potential paracrine effects of local ROS production by infiltrating *Nf1*$^{+/-}$ macrophages on arterial SMC *in vitro*. Under growth-restrictive conditions, *Nf1*$^{+/-}$ SMC exhibit increased proliferation when compared with WT SMC [11,12,26]. To account for this inherent advantage, we compared quiescent *Nf1*$^{+/-}$ and WT SMC incubated with/without H_2O_2 to mimic exposure to vascular oxidative stress. In response to low micromolar concentrations of H_2O_2, *Nf1*$^{+/-}$ SMC exhibited a dose-responsive increase in thymidine incorporation, while higher concentrations of H_2O_2 failed to stimulate *Nf1*$^{+/-}$ SMC proliferation (Fig. 2A). Thymidine incorporation was also dose-responsive in WT SMC, though higher H_2O_2 concentrations were required to induce the same proliferative response and statistical significance was not achieved. Interestingly, WT and *Nf1*$^{+/-}$ SMC viability dropped significantly in response to stimulation with 100 μM H_2O_2 as determined by MTT assay (Fig. 2B). Both low and high concentrations of H_2O_2 preferentially activated Erk in *Nf1*$^{+/-}$ SMC when compared to WT SMC, while Ras-PI-3K activity remained largely unchanged (Fig. 2C). Consistent with decreased cell viability, incubation of *Nf1*$^{+/-}$ SMC with 100 μM H_2O_2 induced the cleavage of caspase-3, an indicator of apoptosis (Fig. 2C). These data suggest that low-level ROS amplifies Erk activity in *Nf1*$^{+/-}$ SMC and potentiates *Nf1*$^{+/-}$ SMC proliferation, while higher concentrations of H_2O_2 may induce *Nf1*$^{+/-}$ SMC apoptosis. The latter is an important observation since SMC apoptosis is a critical step in the development of aortic aneurysms, which is a less common manifestation of NF1 vasculopathy [2,37]. Additionally, we examined *Nf1*$^{+/-}$ and WT SMC for native ROS production in response to incubation with H_2O_2 to identify particular advantages that neurofibromin-deficiency and/or Ras activation might have on ROS production. Quiescent *Nf1*$^{+/-}$ and WT SMC exhibited similar basal ROS production and the addition of H_2O_2 (10 and 100 μM) did not confer additional ROS production in *Nf1*$^{+/-}$ SMC when compared with WT SMC (data not shown). Thus, it is unlikely that *Nf1*$^{+/-}$ SMC produce sufficient quantities of ROS to autonomously induce SMC proliferation and arterial stenosis *de novo*, but may confer an additive effect to local ROS production by infiltrating macrophages.

3.3. NOX2 expression is upregulated in *Nf1*$^{+/-}$ carotid arteries

Previously, we showed that *Nf1*$^{+/-}$ mice develop exaggerated neointimas and excessive remodeling when compared with WT mice, which is largely mediated by neurofibromin-deficient monocytes/macrophages [9,11]. While loss of neurofibromin expression leads to increase Ras signaling and enhances macrophage survival and function, the inciting mechanism(s) leading to rampant SMC proliferation and neointima formation are poorly understood. To examine the potential role of excessive ROS production by *Nf1*$^{+/-}$ macrophages on the vascular wall, we harvested control and ligated arteries from *Nf1*$^{+/-}$ and WT mice 3 days after injury. Prior to tissue harvest, animals were provided dihydroethidium (DHE) via IP injection to detect ROS in the vascular wall. In comparison to control vessels, injured arteries from both genotypes exhibited increase DHE staining indicating an upregulation in ROS production (Fig. 3A). However, injured *Nf1*$^{+/-}$ arteries showed only a modest increase in ROS expression compared to injured WT arteries at this early time point. Next, control and injured carotid arteries from *Nf1*$^{+/-}$ and WT mice were pooled and analyzed for NOX2 expression. Interestingly, control *Nf1*$^{+/-}$ arteries have increased expression of NOX2 compared to WT control arteries (Fig. 3B). In response to arterial ligation, both WT and *Nf1*$^{+/-}$ carotid arteries have enhanced expression of NOX2 compared to genotype-specific controls, though injured *Nf1*$^{+/-}$ arteries exhibited the highest NOX2 expression of all conditions tested. Although examination of an early time point after arterial injury did not detect significant differences in ROS production in the vessel wall between *Nf1*$^{+/-}$ and WT arteries, NOX2 expression in whole artery lysates suggest that neurofibromin regulates the expression of NOX2 and NOX2 expression is increased during early inward arterial remodeling.

3.4. NOX2 activation is required for *Nf1*$^{+/-}$ neointima formation

Excessive ROS production by infiltrating leukocytes in the vascular wall contributes to SMC proliferation and arterial stenosis [34,38] and therapeutic attempts to scavenge excess ROS or interrupt ROS production have proven efficacious in preclinical models of neointima formation [39]. Superoxide production in leukocytes is largely mediated via NADPH oxidase 2 (NOX2), which is activated, in part, by the Ras-dependent kinases Erk and Akt [17,28,40]. Based on our observation that neurofibromin-deficient macrophages produce excessive superoxide and *Nf1*$^{+/-}$ SMC proliferation is dose-responsive to ROS species, we intercrossed *Nf1*$^{+/-}$ mice with *p47*^{phox} knockout mice to understand the role of NOX2 activation in *Nf1*$^{+/-}$ neointima formation. In response to carotid artery ligation, *Nf1*$^{+/-}$

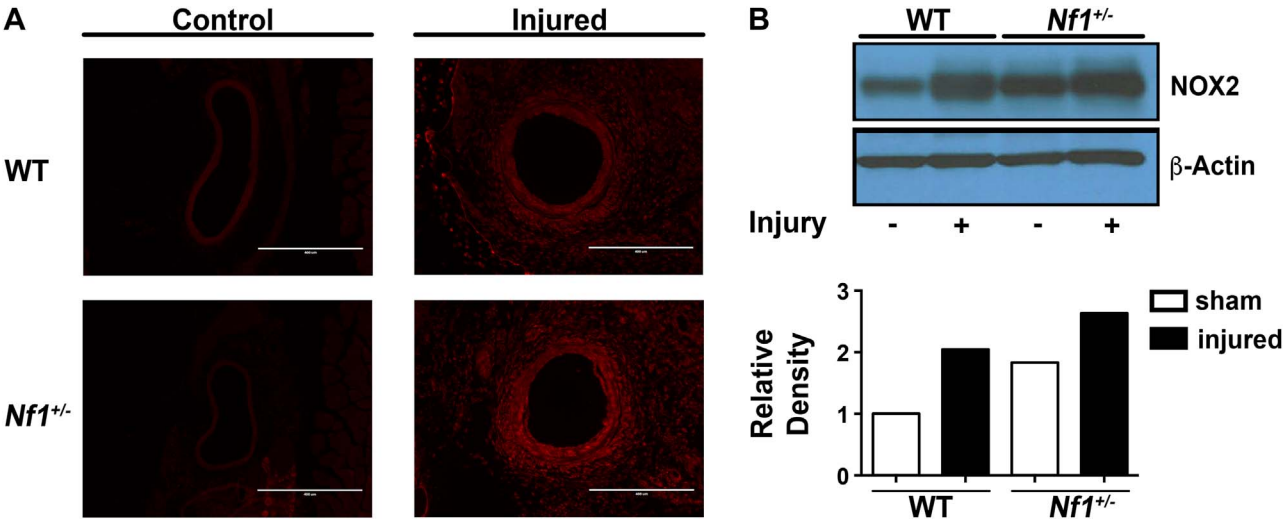

Fig. 3. Loss of neurofibromin enhances NOX2 expression in carotid arteries. Representative photomicrographs (A) and NOX2 expression (B) in control and injured carotid arteries from WT and *Nf1*$^{+/-}$ mice (n=3). A. Fluorescence indicating DHE staining in control and injured WT and *Nf1*$^{+/-}$ arterial cross sections. Scale bars: 400 μm. B. Representative western blot and quantitative densitometry for NOX2 and β-actin in pooled samples from WT *Nf1*$^{+/-}$ carotid arteries.

mice developed an enhanced neointima, which was phenotypically and histologically similar to our previous observations (Fig. 4). Genetic deletion of *p47^{phox}*, a cytosolic component of NOX2 that is required for NOX2 activation, reduced neointima formation in *Nf1$^{+/-}$* mice to levels observed in the background strain. Quantitative analysis of arterial cross sections from each cohort demonstrated a 65% reduction in neointima area and 80% reduction in intima/media (I/M) ratio (Fig. 4B and C). Thus, p47^{phox} expression is required for *Nf1$^{+/-}$* neointima formation and NOX2 activation may be mechanistically responsible for the enhanced ROS production observed in *Nf1* mutant macrophages.

Previously, we utilized *LysM^{cre}* mice to demonstrate that loss of a single *Nf1* gene copy in monocytes/macrophages is sufficient to reproduce the enhanced neointima observed in neurofibromin-deficient mice [11]. In order to specifically interrogate the role of macrophage-specific ROS production in the pathogenesis of *Nf1* arterial stenosis, we intercrossed *Nf1$^{flox/+}$;LysM^{cre}* mice with *gp91$^{flox/flox}$* mice to generate *Nf1$^{flox/+}$;gp91$^{flox/flox}$;LysM^{cre}* mice with specific deletion of both *gp91^{phox}* alleles in *Nf1$^{+/-}$* monocytes and macrophages. gp91^{phox} is the glycosylated membrane-bound component of NOX2 and is required for electron transfer to molecular oxygen in the generation of superoxide [41]. Cre^{+} and Cre^{-} mice were subjected to carotid artery ligation to induce neointima formation. Lineage-restricted inactivation of a single *Nf1* allele in myeloid cells alone was sufficient to induce a robust neointima that is an exact phenocopy of neointimas observed in *Nf1$^{+/-}$* mice after arterial injury (Fig. 5). In contrast, genetic deletion of both *gp91^{phox}* alleles in *Nf1$^{flox/+}$;LysM^{cre}* mice resulted in a 75% reduction in neointima formation after arterial injury when compared to *Nf1$^{flox/+}$;LysM^{cre}* mice (Fig. 5B and C). Collectively, these data provide genetic evidence that the presence and activation of NOX2 is necessary for *Nf1$^{+/-}$* neointima formation and limiting ROS production may be a viable therapeutic target for the prevention and/or treatment of NF1 arterial stenosis.

3.5. Apocynin inhibits neointima formation in *Nf1$^{+/-}$* mice

The proximity of invading *Nf1$^{+/-}$* macrophages to vascular wall SMC and their propensity for superoxide production may be pathologically linked to neointima formation in *Nf1$^{+/-}$* mice.

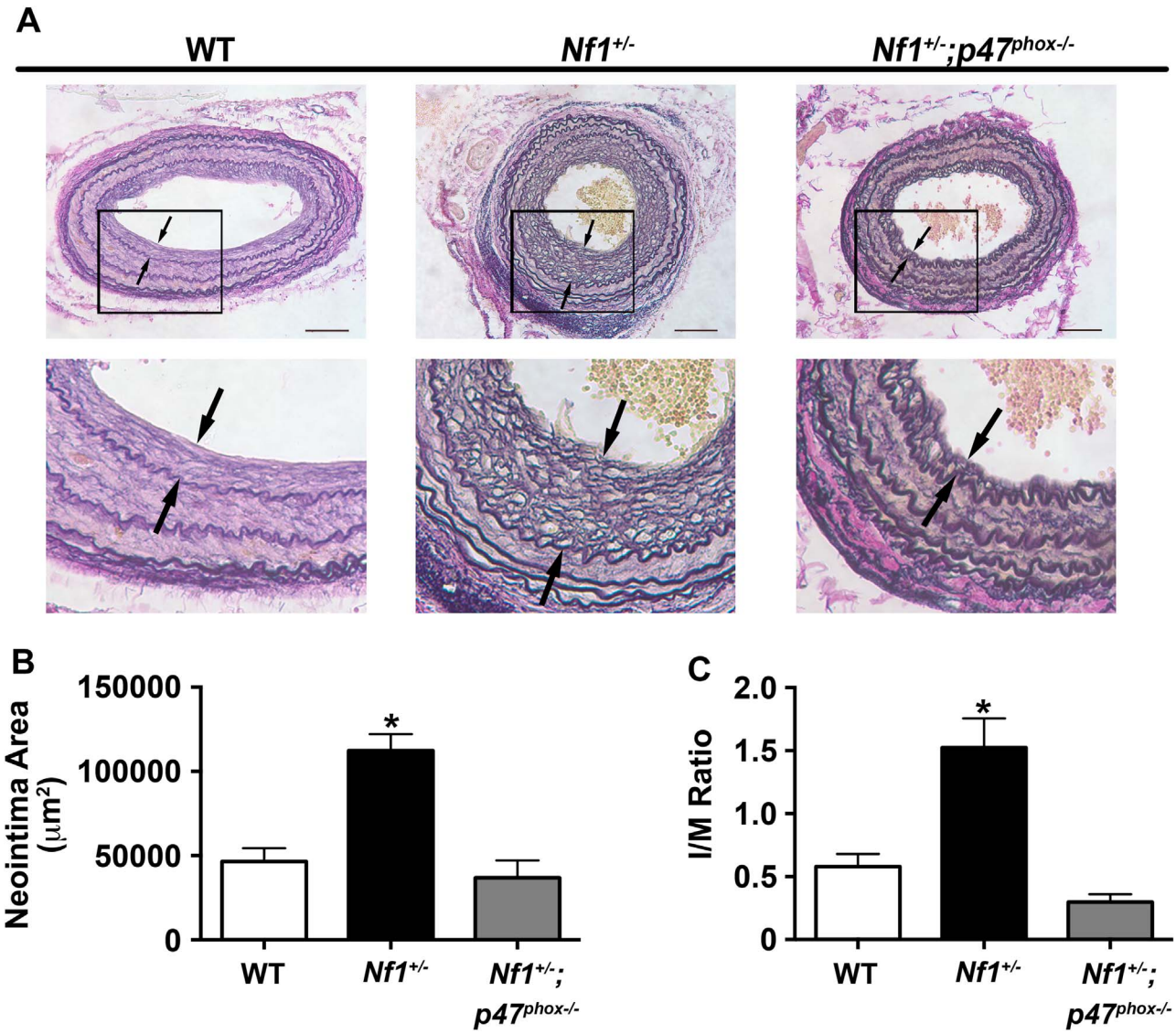

Fig. 4. Genetic deletion of *p47^{phox}* inhibits *Nf1$^{+/-}$* neointima formation. Representative photomicrographs (A) and quantification of neointima area (B and C) of injured carotid arteries from WT, *Nf1$^{+/-}$*, and *Nf1$^{+/-}$;p47$^{phox-/-}$* mice. A. Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified below. Scale bars: 100μm. B and C. Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from WT, *Nf1$^{+/-}$*, and *Nf1$^{+/-}$;p47$^{phox-/-}$* mice. Data represent mean neointima area or I/M ratio ± SEM, $n=8$–11. *$P<0.001$ for WT and *Nf1$^{+/-}$;p47$^{phox-/-}$* mice versus *Nf1$^{+/-}$* mice.

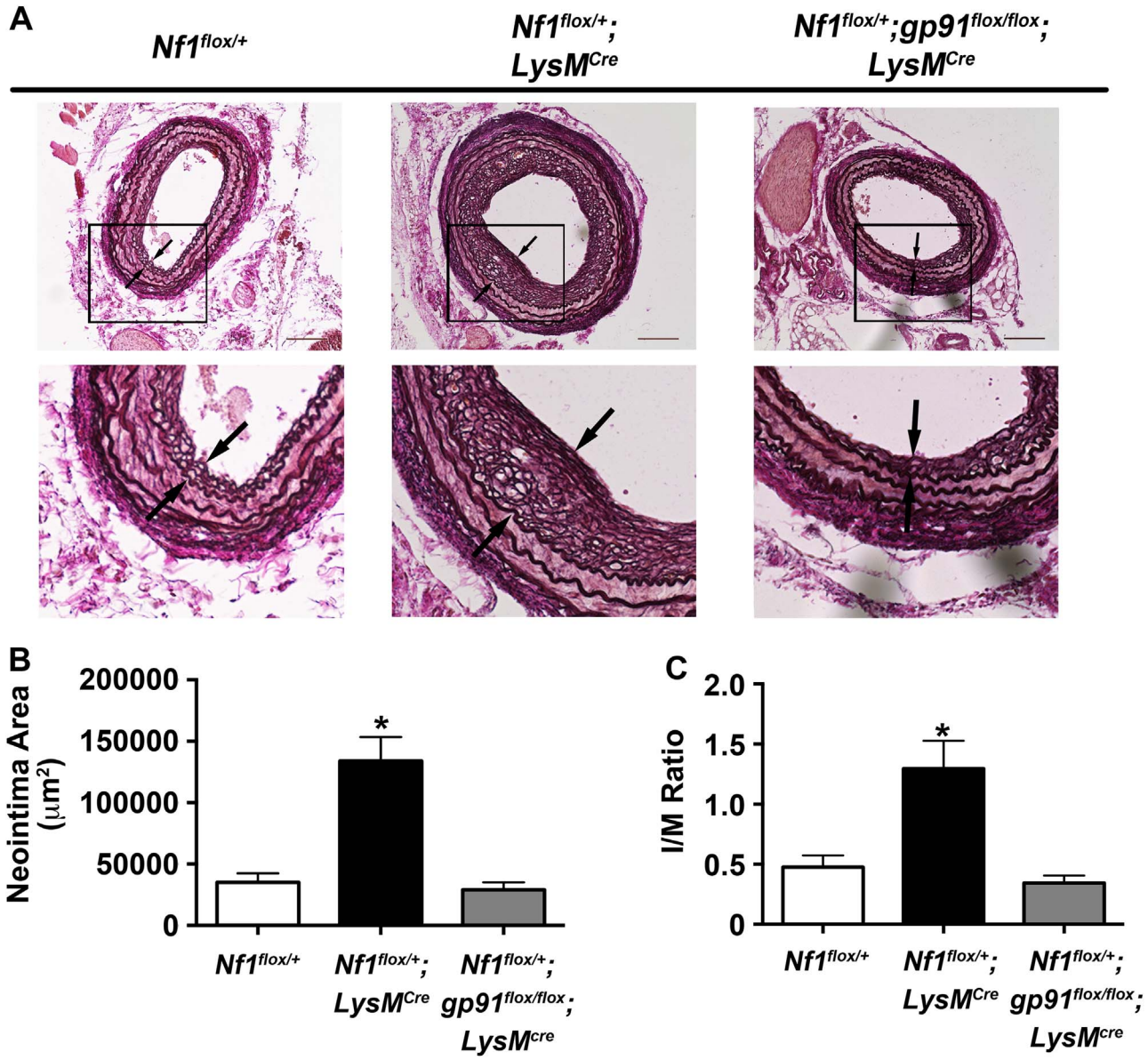

Fig. 5. Lineage restricted deletion of *gp91*^{phox} in myeloid cells inhibits *Nf1*$^{+/-}$ neointima formation. Representative photomicrographs (A) and quantification of neointima area (B and C) of injured carotid arteries from WT, *Nf1*$^{flox/+}$;*LysM*^{cre}, and *Nf1*$^{flox/+}$;*gp91*$^{flox/flox}$;*LysM*^{cre} mice. A. Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified below. Scale bars: 100 μm. B and C. Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from WT, *Nf1*$^{flox/+}$;*LysM*^{cre}, and *Nf1*$^{flox/+}$;*gp91*$^{flox/flox}$;*LysM*^{cre} mice. Data represent mean neointima area or I/M ratio ± SEM, $n = 10$–12. *$P < 0.001$ for WT, and *Nf1*$^{flox/+}$;*gp91*$^{flox/flox}$;*LysM*^{cre} mice versus *Nf1*$^{flox/+}$;*LysM*^{cre}.

Sequestering ROS production provides an attractive therapeutic option since anti-oxidants are well tolerated and may be particularly effective in NF1 patients with evidence of oxidative stress. The antioxidant apocynin is intriguing since it binds the p47 subunit in the cytosol and interferes with NOX2 activation [42–44]. Therefore, we subjected *Nf1*$^{+/-}$ and WT mice to carotid artery ligation to induce arterial stenosis and provided drinking water containing or lacking apocynin (100 mg/kg/day) for 28 days until the arteries were harvested and analyzed for neointima formation. In response to carotid artery injury, WT mice developed a modest neointima while *Nf1*$^{+/-}$ mice developed a severe arterial stenosis, which was similar to our previous observations (Fig. 6). In contrast, daily administration of apocynin reduced *Nf1*$^{+/-}$ neointima area and I/M ratio by 75% in comparison with control *Nf1*$^{+/-}$ mice (Fig. 6A and B). WT mice experienced a modest, but non-significant reduction in neointima formation, which is likely due to their resistance to neointima formation.

3.6. NF1 patients have increased pro-inflammatory monocytes and evidence of chronic oxidative stress

Loss of *Nf1* may increase the susceptibility of whole organisms and primary cells to oxidative stress, and previous studies have suggested interplay between chronic oxidative stress and inflammation in tissue derived from NF1 patients. However, evidence of oxidative stress has not been demonstrated in persons with NF1 [8,45–47]. Therefore, we isolated peripheral blood MNCs to examine them for evidence of oxidative stress. In comparison with age- and sex-matched controls, total monocyte count was elevated in asymptomatic NF1 patients. Examination of monocyte subpopulation frequency showed that some healthy persons with NF1 had a substantial increase in the frequency of a monocyte subpopulation ($CD14^+CD16^{++}$) associated with inflammatory conditions and oxidative stress [48–50] (Fig. 7 A and B). The mean frequency of $CD14^+CD16^{++}$ monocytes in the NF1 cohort was

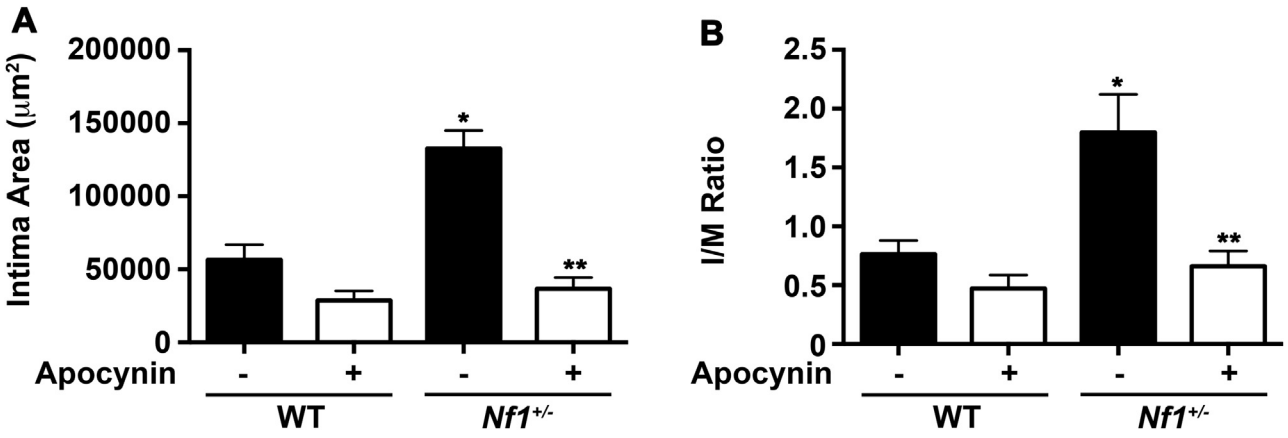

Fig. 6. Apocynin, a NOX2 inhibitor, reduces *Nf1*$^{+/-}$ neointima formation. Quantification of neointima area (A) and I/M ratio (B) of injured carotid arteries from WT and *Nf1*$^{+/-}$ mice treated with apocynin (100 mg/kg/day) or water. Data represent mean neointima area or I/M ratio ± SEM, $n=8$–10. *$P<0.001$ for WT versus *Nf1*$^{+/-}$ mice treated with water. **$P<0.001$ for *Nf1*$^{+/-}$ mice treated with apocynin versus *Nf1*$^{+/-}$ mice treated with water. No statistical difference was observed between WT mice treated with apocynin and WT mice treated with water.

2.5 times higher than the mean frequency of $CD14^+CD16^{++}$ monocytes in the control cohort (40.8 ± 14 vs. 15.73 ± 4.3 cells/μL, $P=0.06$). Within the NF1 cohort, we observed that some NF1 patients exhibited a significant elevation in the frequency of circulating $CD14^+CD16^{++}$ monocytes, which may cause a positive skew in the sample distribution. To determine if the sample distribution in these two cohorts could occur by random chance, we performed an *F*-test of variance. This secondary analysis revealed a significant difference in $CD14^+CD16^{++}$ monocyte frequency variance between NF1 and control patients ($P<0.001$). Monocyte subpopulation frequencies must be interpreted in relationship to controls since normal values do not presently exist. While nearly 60% of healthy controls had a $CD14^+CD16^{++}$ monocyte frequency of less than 10,000 cells/μL in their peripheral blood, less than 45% of healthy persons with NF1 met the same cutoff value. In fact, the highest $CD14^+CD16^{++}$ monocyte frequency observed in persons with NF1 were 2–3 fold higher than the highest observed frequencies in control patients (Fig. 7B). In comparison, the frequency of patrolling or "classical" monocytes did not differ between control and NF1 patients Fig. 7C. Since $CD14^{++}CD16^-$ classical

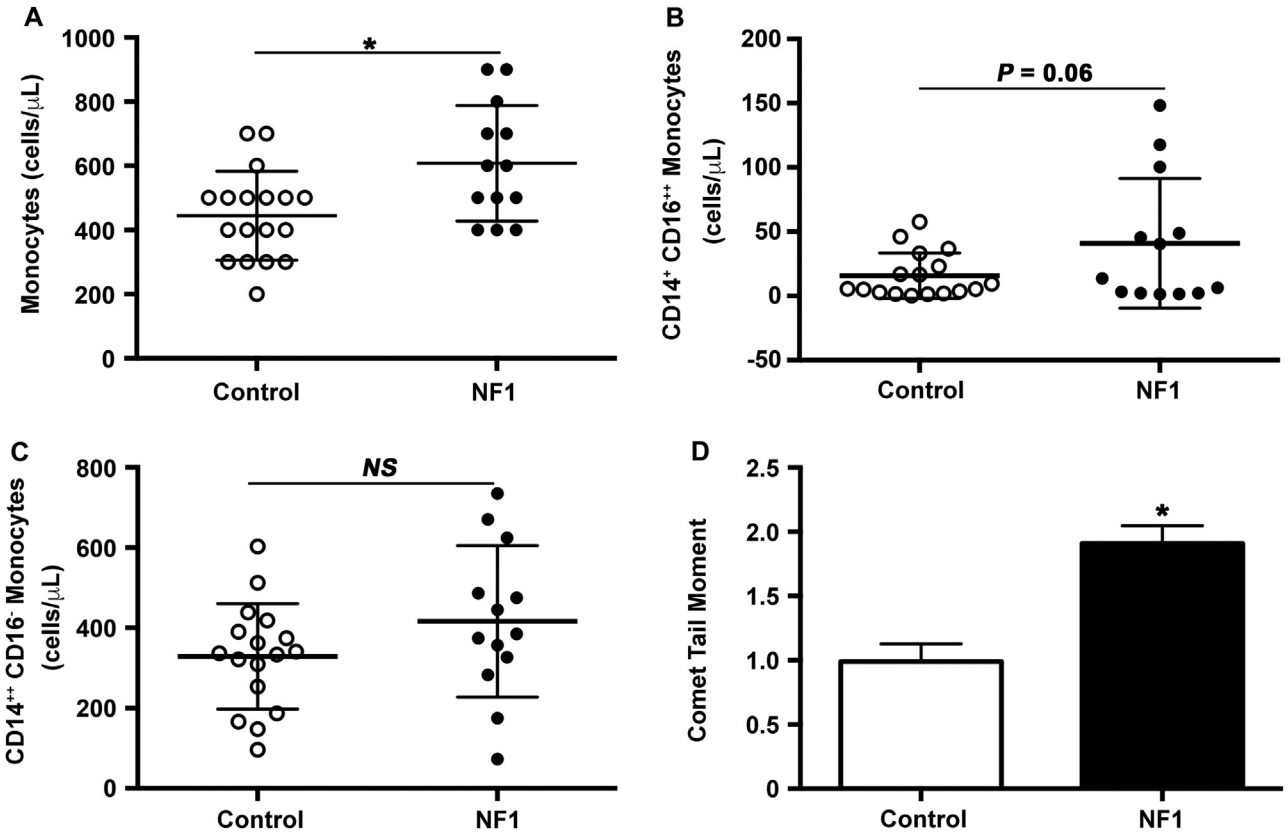

Fig. 7. NF1 patients have evidence of chronic inflammation and oxidative stress. Quantification of total monocyte count (A), $CD14^+CD16^{++}$ monocyte count (B), and peripheral blood MNC comet tail moment (C) in NF1 patients and age-, sex-matched controls. A. Data represent total monocyte count (cells/μL) for NF1 patients ($n=13$) and controls ($n=18$). $P<0.01$ for NF1 patients versus control patients. B. Data represent $CD14^+CD16^{++}$ monocytes (cells/μL) for NF1 patients ($n=13$) and controls ($n=17$). C. Data represent $CD14^{++}CD16^-$ monocytes (cells/μL) for NF1 patients ($n=13$) and controls ($n=17$). D. Data represent mean comet tail moment ± S.D. for NF1 patients ($n=10$) versus controls ($n=28$). $P<0.05$ for NF1 versus control patients.

monocytes arise from the bone marrow and give rise to $CD14^{++}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ non-classical monocytes, this data suggests that the increased frequency of $CD14^{+}CD16^{++}$ monocytes is the result of signaling inputs outside the bone marrow compartment and do not arise from alterations in developmental maturation [50].

Next, we examined peripheral blood MNCs for evidence of oxidative stress using a modified comet assay to analyze for oxidative DNA damage. Monocytes isolated from NF1 patients displayed a 2-fold increase in oxidative DNA damage when compared with control patients (Fig. 7D Thus, NF1 patients have evidence of chronic oxidative stress and inflammation, which may predispose them to premature and/or severe forms of cardiovascular disease.

4. Discussion

Despite a high propensity for severe forms of cardiovascular disease, tailored therapies for NF1 patients with vasculopathy are nonexistent. The lack of a concise therapeutic approach to NF1 vasculopathy is largely due to a poor understanding of disease pathogenesis and latency in disease presentation. To date, most therapeutic studies have targeted downstream Ras kinases as their activity is deregulated and amplified in persons with NF1. While Ras pathway inhibitors are mechanistically plausible for the treatment of cardiovascular manifestations in NF1 patients, long-term treatment of cardiovascular diseases with Mek-Erk or Ras-PI-3K inhibitors is not practical due to the necessity of this highly conserved pathway in normal cell growth and differentiation. Thus, the need for highly efficacious and well-tolerated compounds for persons with NF1 vasculopathy must leverage the unique biochemistry found in neurofibromin-deficient tissue while maintaining a favorable side-effect profile.

Emerging evidence suggests that oxido-reductive balance is disturbed in active Ras mutants and neurofibromin-deficient tissues and humans. The interaction between ROS and Ras is multifaceted, but reactive oxygen and nitrogen species have been identified as upstream modulators and downstream targets of several Ras kinases [13]. Active Ras mutations or upregulation of Ras signaling leads to increased expression of NADPH oxidases to enhance ROS production in multiple cell types [17,51–53]. Ras activation through growth factor binding of various receptor tyrosine kinases may cooperate with small molecule modulators, such as ROS, to enhance phosphorylation of the downstream kinases Erk and Akt [54]. In turn, inhibition of ROS production appears to decrease Erk and Akt activity so that signaling feedback between ROS and Ras is critical for cell homeostasis [55–57]. The physiologic interaction of ROS and $p21^{Ras}$ may turn pathologic in the setting of neurofibromin-deficiency as evidenced by recent studies. Oligodendrocytes containing either inactivating *Nf1* or active *HRAS* mutations produce excessive ROS leading to disruption of local endothelial tight junctions and increased vascular permeability, which is restored with the administration of the antioxidant N-acetyl cysteine [23]. Neurofibromin may also express a more direct relationship with mitochondrial ROS production via protein kinase A (PKA). Mutations in the *Drosophila Nf1* homolog increased mitochondrial ROS production, shortened lifespan, and sensitized flies to oxidative stress [22]. Overexpression of PKA restored a normal response to oxidative stress in *Nf1* mutant *Drosophila* [58]. Conversely, overexpression of neurofibromin in *Drosophila* provided resistance to oxidant injury and prolonged mean lifespan. Here, we show that superoxide production by differentiated macrophages is directly regulated by neurofibromin via a gene-dosage dependent mechanism.

In support of neurofibromin's role in ROS production, we provide the first evidence of chronic oxidative stress in NF1 patients, as demonstrated by increased comet tail moment, an indicator of oxidative DNA damage [59]. Recent studies suggest that neurofibromin also regulates cell cycle and DNA repair pathways; therefore, loss of neurofibromin may prevent normal DNA repair to occur and may be an alternative explanation for the enhanced oxidative DNA damage [60]. However, measurement of direct DNA damage did not differ between NF1 and control patients (data not shown). Regardless, the implications of these findings may be broad-based since excessive ROS production and oxidative DNA damage may participate in several manifestations of NF1, including cancer, impaired learning and cognition, and musculoskeletal diseases. Further, modulating ROS production is an attractive therapeutic alternative for NF1 patients and may have additive effects since neurofibromin restrains ROS production directly and indirectly.

Monocytes and macrophages appear to play a critical role in the pathogenesis of NF1 vasculopathy. Lineage-restricted inactivation of *Nf1* in myeloid cells leads to a pro-inflammatory monocyte profile in mice and is sufficient to recapitulate the arterial stenosis phenotype observed in persons with NF1 [11]. *Nf1* mutant macrophages are readily recruited to sites of vascular injury and the excessive ROS production by $Nf1^{+/-}$ macrophages appears to promote a proliferative SMC response *in vitro* and *in vivo*. Interestingly, PMA elicited more ROS in *Nf1* mutant macrophages as compared to WT macrophages, which was effectively blocked by co-incubation with inhibitors of Ras. PMA is a potent stimulus for NADPH via Ras-Erk dependent and independent mechanisms and may explain the exaggerated response of $Nf1^{+/-}$ and $Nf1^{-/-}$ macrophages to PMA [61–64]. Increased production of ROS may propagate a pro-survival macrophage phenotype and increase the generation and secretion of pro-inflammatory cytokines, although this has yet to be demonstrated [65]. Neurofibromin's role in regulating monocyte/macrophage-mediated ROS is further emphasized by the observation that apocynin significantly inhibited neointima formation in $Nf1^{+/-}$ mice while having little effect on WT neointima formation. Although apocynin is generally recognized as a nonspecific antioxidant in vascular wall cells, Heumuller et al. concluded that apocynin dimerizes in myeloperoxidase-expressing cells, including macrophages, and suppresses superoxide production in leukocytes by inhibiting the binding of the cytosolic $p47^{phox}$ subunit to the transmembrane NOX2 complex [42]. Apocynin's dual roles as an antioxidant and specific inhibitor of NOX2 activity likely contribute to the reduced neointima formation observed in $Nf1^{+/-}$ mice. These observations are supported by our findings that genetic deletion of $p47^{phox}$ or myeloid cell specific inactivation of $gp91^{phox}$ inhibited neointima formation in $Nf1^{+/-}$ mice. Further, the activation of $p47^{phox}$ is highly regulated by the Ras dependent kinases Erk and Akt, which is required for NOX2 activation and superoxide production in circulating phagocytes [66–68]. Directly targeting NOX2 for the prevention and/or treatment of NF1-related arterial stenosis may not be viable as NOX2 is critical for respiratory burst and phagocytosis, though clinical trails of small molecule NOX inhibitors are forthcoming [40].

Antioxidants, on the other hand, have yielded promising results in multiple preclinical models of arterial stenosis, but human clinical trials have demonstrated only a modest effect [69–71]. Inhibition of ROS production or sequestering its activity via antioxidant therapy may provide a greater beneficial effect in patients with active Ras mutations, including NF1, than that observed in the general population since small perturbations in kinase activity precipitate a dramatic increase in downstream signaling. For example, anti-oxidant therapy significantly reduced vascular wall ROS in $Nf1^{+/-}$ aortas exposed to angiotensin II (AngII) while having minimal effect on ROS in WT aortas [72]. The preferential effect of antioxidant therapy in $Nf1^{+/-}$ mice may be explained by the high numbers of macrophages observed in $Nf1^{+/-}$ aortas exposed to

AngII resulting in an abundance of oxidant species. Interestingly, low dose simvastatin, an HMG-CoA reductase inhibitor with antioxidant properties, effectively blocked ROS production in *Nf1*$^{+/-}$ aortas exposed to AngII with only a modest treatment effect observed in WT aortas exposed to AngII. While the pleiotropic effects of statins are widely studied, their function as an inhibitor of prenylation has made them an attractive therapy for NF1 patients since prenylation is required for Ras activation [73]. The ability of statins to modulate Ras activity and scavenge oxidative species may cooperate to limit the production and local concentration of ROS within the vascular wall. Statins have proven beneficial for a variety of NF1 manifestations including cognitive deficits, behavioral impairment, bone dysplasia and healing in preclinical models, but randomized trials in NF1 patients have yielded mixed results [11,74–77]. To date, no clinical trials for NF1 vasculopathy have been performed.

In summary, our study identifies a novel role for neurofibromin signaling in the generation of reactive oxygen species and provides genetic and pharmacologic evidence that excessive ROS is linked to NF1 vasculopathy. Further, we provide the first human data to suggest that NF1 patients experience chronic oxidative stress. As neurofibromin-deficient myeloid cells are critical cellular mediators of multiple manifestations of NF1, our findings provide a framework for interrogating ROS in NF1 biology and the rational design of clinical trials using antioxidants for NF1 vasculopathy.

Conflict of interest

The authors have declared that no conflict of interest exists.

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ORIGINAL ARTICLE

Nf1$^{+/-}$ monocytes/macrophages induce neointima formation via CCR2 activation

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Abstract

Persons with neurofibromatosis type 1 (NF1) have a predisposition for premature and severe arterial stenosis. Mutations in the *NF1* gene result in decreased expression of neurofibromin, a negative regulator of p21^{Ras}, and increases Ras signaling. Heterozygous *Nf1* (*Nf1*$^{+/-}$) mice develop a marked arterial stenosis characterized by proliferating smooth muscle cells (SMCs) and a predominance of infiltrating macrophages, which closely resembles arterial lesions from NF1 patients. Interestingly, lineage-restricted inactivation of a single *Nf1* allele in monocytes/macrophages is sufficient to recapitulate the phenotype observed in *Nf1*$^{+/-}$ mice and to mobilize proinflammatory CCR2+ monocytes into the peripheral blood. Therefore, we hypothesized that CCR2 receptor activation by its primary ligand monocyte chemotactic protein-1 (MCP-1) is critical for monocyte infiltration into the arterial wall and neointima formation in *Nf1*$^{+/-}$ mice. MCP-1 induces a dose-responsive increase in *Nf1*$^{+/-}$ macrophage migration and proliferation that corresponds with activation of multiple Ras kinases. In addition, *Nf1*$^{+/-}$ SMCs, which express CCR2, demonstrate an enhanced proliferative response to MCP-1 when compared with WT SMCs. To interrogate the role of CCR2 activation on *Nf1*$^{+/-}$ neointima formation, we induced neointima formation by carotid artery ligation in *Nf1*$^{+/-}$ and WT mice with genetic deletion of either *MCP1* or *CCR2*. Loss of MCP-1 or CCR2 expression effectively inhibited *Nf1*$^{+/-}$ neointima formation and reduced macrophage content in the arterial wall. Finally, administration of a CCR2 antagonist significantly reduced *Nf1*$^{+/-}$ neointima formation. These studies identify MCP-1 as a potent chemokine for *Nf1*$^{+/-}$ monocytes/macrophages and CCR2 as a viable therapeutic target for NF1 arterial stenosis.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder affecting 1 in 3000 persons and is the result of inactivating mutations in the *NF1* tumor suppressor gene. Neurofibromin, the protein product of *NF1*, functions as a GTPase activating protein for p21^{ras} (Ras) and accelerates the slow, intrinsic hydrolysis of active Ras-GTP to its inactive diphosphate conformation (1). Inherited mutations of *NF1* affect a single gene copy and result in disease with complete penetrance and a broad range of clinical features.

Cardiovascular disease represents a common, yet understudied, manifestation of NF1 that contributes to the early mortality observed in this patient population (2). NF1 vasculopathy

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primarily affects the arterial network with a strong predilection for the renal artery and proximal branches of the carotid artery. The exact frequency of vasculopathy in NF1 patients is unknown; however, multiple case series and large patient cohorts suggest the prevalence of arterial disease approaches 10% (3–7). The insidious and progressive clinical presentation of these lesions in early adulthood likely places the true incidence much higher. In fact, histologic evidence of cardiovascular disease was identified in nearly 50% of young adults with NF1 in one case series (8), whereas a comprehensive review of 3253 death certificates revealed a diagnosis of vasculopathy was listed 7.2 times more frequently than expected among NF1 patients less than 30 years of age at the time of death (4).

Arterial lesions associated with NF1 are characterized by smooth muscle cell (SMC) hyperplasia, leukocyte infiltration and arterial remodeling leading to vasoocclusion and tissue ischemia (2,9–11). Previously, we developed a mouse model of NF1 arterial stenosis using *Nf1* heterozygous ($Nf1^{+/-}$) mice that completely recapitulates the human phenotype (12). Although neointima formation is the result of complex interactions between vascular wall cells and circulating leukocytes, we showed that loss of a single *Nf1* allele in bone marrow cells is both necessary and sufficient to induce arterial stenosis (13). These results were somewhat surprising, because neurofibromin-deficient SMC have increased proliferation and migration in response to multiple growth factors and $Nf1^{+/-}$ macrophages (13,14). Neurofibromin-deficient macrophages are the dominant hematopoietic cell within the neointima of $Nf1^{+/-}$ mice and likely secrete cytokines and chemokines that stimulate SMC proliferation and migration (13,15). Thus, it is plausible that loss of neurofibromin in bone marrow and circulating hematopoietic cells, particularly monocytes and macrophages, may predispose NF1 patients to develop exaggerated responses to acute and chronic inflammation or insult. Emerging evidence in NF1 patients is supportive of this hypothesis. Asymptomatic NF1 patients have increased circulating proinflammatory cytokines and monocytes ($CD14^+CD16^+$) in the peripheral blood compared with controls (13). Similar to NF1 patients, $Nf1^{+/-}$ mice have increased circulating $Ly6C^{hi}CCR2^+$ monocytes, which are the murine correlate of human proinflammatory monocytes (15,16). Murine $Ly6C^{hi}CCR2^+$ leukocytes are primitive bone-marrow-derived monocytes that are actively recruited to sites of inflammation and differentiate into macrophages and inflammatory dendritic cells (17,18). In support of our hypothesis that neurofibromin regulates inflammatory cascades in circulating leukocytes, we recently showed that loss of a single *Nf1* gene copy in myeloid cells was sufficient to mobilize $Ly6C^{hi}CCR2^+$ monocytes and induce arterial stenosis following carotid artery ligation (15). Interestingly, genetic deletion of both *Nf1* gene copies in myeloid cells alone resulted in a 4-fold increase in circulating $Ly6C^{hi}CCR2^+$ monocytes and nearly complete arterial occlusion following carotid ligation (15).

Based on these observations, we generated compound mutant mice to test the hypothesis that CCR2 activation is critical for $Nf1^{+/-}$ macrophage recruitment to sites of vascular injury and necessary for $Nf1^{+/-}$ neointima formation. Further, we sought to understand how CCR2 activation by its primary ligand, monocyte chemotactic protein-1 (MCP-1/CCL2), mediates neurofibromin-deficient macrophage function and SMC proliferation. Finally, we use our murine model system to test the efficacy of a potent and specific inhibitor of the CCR2 receptor as a potential therapeutic intervention in the treatment of NF1 arterial stenosis.

Results

MCP-1 is a potent chemokine for $Nf1^{+/-}$ macrophages and SMC

MCP-1 is a monomeric polypeptide anchored to the endothelial monolayer of blood vessels and secreted by SMC and circulating leukocytes (19,20). The chemotactic properties of MCP-1 are primarily mediated through its activation of the CCR2 receptor (19,21–23). Importantly, the expression of CCR2 in the cardiovascular system is restricted to endothelial cells (ECs), SMC and monocytes/macrophages and is not a mediator of granulocyte chemotaxis (24–26). Therefore, we derived $Nf1^{+/-}$ and WT macrophages from $Nf1^{+/-}$ and WT mice to assess their functional response to MCP-1 stimulation. At baseline, $Nf1^{+/-}$ macrophages demonstrate a 2-fold increase in migration, but do not exhibit enhanced proliferation. In response to MCP-1 incubation, $Nf1^{+/-}$ macrophages exhibited a dose-dependent increase in chemotaxis and proliferation when compared with WT macrophages (Fig. 1A and B). Although MCP-1 also increased WT macrophage migration and proliferation in a dose-dependent manner, MCP-1 stimulated a 2- to 3-fold increase in $Nf1^{+/-}$ macrophage migration and proliferation when compared with WT macrophages at the same concentration of MCP-1. Interestingly, analysis of culture media from growth-arrested $Nf1^{+/-}$ and WT macrophages revealed an increased concentration of MCP-1 in the media of cultured $Nf1^{+/-}$ macrophages when compared with WT macrophage media (Fig. 1C). Cell counts of growth-arrested macrophages did not differ between genotypes (data not shown). Next, we analyzed neurofibromin-regulated Ras kinase activity to determine which pathway may be preferentially activated in in $Nf1^{+/-}$ macrophages in response to MCP-1 (Fig. 1D). While phosphorylation of both Erk and Akt was demonstrated in $Nf1^{+/-}$ macrophages incubated with MCP-1, the increase in p-Erk/Erk ratio did not differ between $Nf1^{+/-}$ and WT macrophages incubated with MCP-1 and likely represents an increase in Erk activity that is independent of neurofibromin expression. Akt phosphorylation, on the other hand, was dramatically higher in $Nf1^{+/-}$ macrophages stimulated with MCP-1 when compared with WT macrophages stimulated with MCP-1. Thus, $Nf1^{+/-}$ macrophages exhibit an exaggerated response to MCP-1 stimulation that corresponds with preferential activation of the PI3-K–Akt pathway.

Infiltrating macrophages secrete growth factors and cytokines to induce SMC proliferation and inward remodeling, which are hallmarks of arterial stenosis. Although genetic deletion of *Nf1* in SMC is not required for $Nf1^{+/-}$ neointima formation, $Nf1^{+/-}$ SMCs demonstrate enhanced proliferation in response to multiple growth factors and co-incubation with $Nf1^{+/-}$ and WT macrophages (14,15,27). Based on the observation that $Nf1^{+/-}$ macrophages exhibit increased production of MCP-1 and that SMC express CCR2, we isolated SMC from WT, $Nf1^{+/-}$ and $Nf1^{+/-}$; $CCR2^{-/-}$ mice to assess their proliferative response to MCP-1. Similar to previous published reports, WT SMC showed a modest proliferative response to MCP-1 (28–31) (Fig. 2A). While $Nf1^{+/-}$ SMCs are more proliferative at baseline, $Nf1^{+/-}$ SMC exhibited a nearly 2-fold increase in proliferation in response to MCP-1 stimulation when compared with untreated $Nf1^{+/-}$ SMC. MCP-1 expression in cultured $Nf1^{+/-}$ SMC and secretion into growth media was similar to WT SMC (data not shown). Genetic deletion of CCR2 in $Nf1^{+/-}$ SMC completely abolished the proliferative response mediated by MCP-1 and demonstrates that MCP-1 is primarily activating CCR2 on $Nf1^{+/-}$ SMC (Fig. 2A). Examination of Ras-dependent kinase activity revealed a profound increase in Erk signaling in $Nf1^{+/-}$ SMC stimulated with MCP-1 when

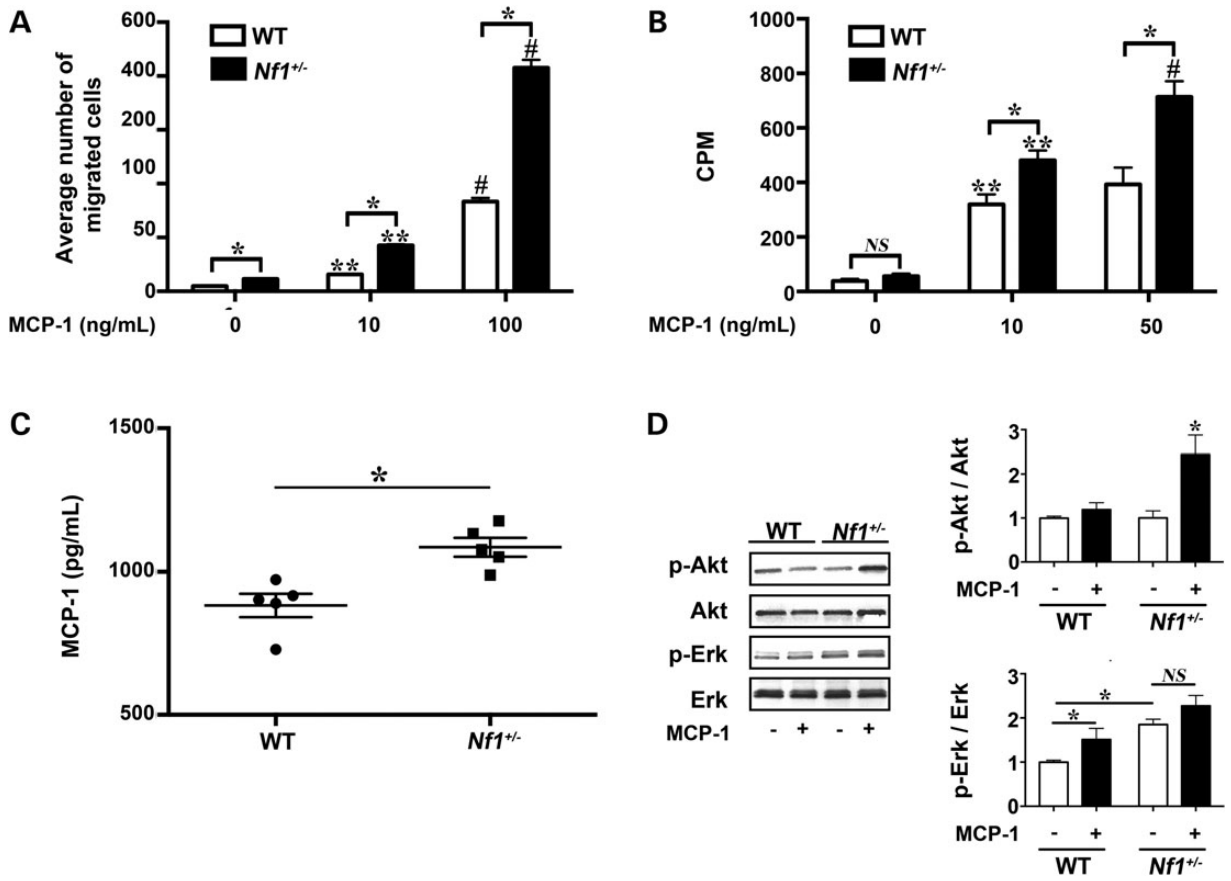

Figure 1. MCP-1 enhances $Nf1^{+/-}$ macrophage function. WT (white bars) and $Nf1^{+/-}$ (black bars) macrophage migration and proliferation, in response to MCP-1. (**A**) Data represent average number of migrated cells per HPF ± SEM, $n = 5$. *$P < 0.001$ for WT versus $Nf1^{+/-}$ macrophages at indicated concentration of MCP-1. **$P < 0.001$ for unstimulated WT and $Nf1^{+/-}$ macrophages versus MCP-1 (10 ng/ml) stimulated WT and $Nf1^{+/-}$ macrophages. #$P < 0.0001$ for MCP-1 (10 ng/ml) stimulated WT and $Nf1^{+/-}$ macrophages versus MCP-1 (100 ng/ml) stimulated WT and $Nf1^{+/-}$ macrophages. (**B**) Data represent thymidine incorporation reported as mean counts per minute (cpm) ± SEM, $n = 5$. *$P < 0.001$ for WT versus $Nf1^{+/-}$ macrophages at indicated concentration of MCP-1. **$P < 0.001$ for unstimulated WT and $Nf1^{+/-}$ macrophages versus MCP-1 (10 ng/ml) stimulated WT and $Nf1^{+/-}$ macrophages. #$P < 0.001$ for MCP-1 (10 ng/ml) stimulated $Nf1^{+/-}$ macrophages versus MCP-1 (50 ng/ml) stimulated $Nf1^{+/-}$ macrophages. (**C**) Data represent MCP-1 concentration reported as pg/ml ± SEM, $n = 5$. *$P < 0.01$ for WT versus $Nf1^{+/-}$ macrophage-conditioned media. (**D**) Representative western blots of Akt and Erk phosphorylation in WT and $Nf1^{+/-}$ macrophages treated with/without MCP-1 (10 ng/ml), $n = 4$. Quantitative densitometry ± SEM is reported as ratio of phosphorylated-Akt to total Akt or phosphorylated-Erk to total Erk density and corrected to unstimulated WT macrophages. *$P < 0.001$ for pAkt/Akt ratio in all conditions versus $Nf1^{+/-}$ macrophages stimulated with MCP-1. *$P < 0.01$ for pErk/Erk ratio for unstimulated WT macrophages versus MCP-1 stimulated WT macrophages and unstimulated $Nf1^{+/-}$ macrophages.

compared with unstimulated $Nf1^{+/-}$ SMC (Fig. 2B). MCP-1 did not alter Akt activity in $Nf1^{+/-}$ SMC (data not shown). Based on the substantial increase in Erk activity in response to MCP-1, we incubated $Nf1^{+/-}$ and WT SMC with MCP-1 (10 ng/ml) in the presence or absence of PD0325901, a potent inhibitor of Mek–Erk signaling. At low nanomolar concentrations of PD0325901, MCP-1 failed to evoke a proliferative response in *Nf1*+/– SMC (Fig. 2C). Not surprisingly, blockade of Erk signaling also reduced WT SMC proliferation and strengthens the argument that Erk activation is critical for MCP-1 elicited SMC proliferation. Collectively, these data suggest that $Nf1^{+/-}$ SMC exhibit enhanced proliferation in response to MCP-1 via Erk activation.

Genetic deletion of MCP-1 or CCR2 abrogates $Nf1^{+/-}$ neointima formation

Based on our observations that MCP-1 is a potent stimulus for $Nf1^{+/-}$ macrophage and SMC, we sought to interrogate the role of MCP-1/CCR2 signaling in the pathogenesis $Nf1^{+/-}$ arterial stenosis. $Nf1^{+/-}$ mice were intercrossed with $MCP1^{-/-}$ mice to generate compound mutant $Nf1^{+/-}$;$MCP1^{-/-}$ mice. WT, $MCP1^{-/-}$, $Nf1^{+/-}$ and $Nf1^{+/-}$; $MCP1^{-/-}$ mice underwent surgical ligation of the right common carotid artery to induce neointima formation. In response to arterial injury, WT mice developed a modest neointima, whereas $Nf1^{+/-}$ mice developed a robust neointimal layer after arterial injury (Fig. 3). Genetic deletion of MCP-1 completely inhibited neointima formation in $Nf1^{+/-}$ mice. Morphometric analysis of serial cross sections revealed a 70% reduction in neointima area (Fig. 3B) and a 55% reduction in intima/media (I/M) ratio (Fig. 3C). Corresponding with the reduced neointima in $Nf1^{+/-}$ mice lacking MCP-1 expression, Mac-3+ macrophage staining in the neointimas of $Nf1^{+/-}$; $MCP1^{-/-}$ mice were significantly reduced when compared with $Nf1^{+/-}$ neointimas (10.4 ± 3.0% versus 17.3 ± 3.4% of total cell number, $P = 0.1$; Fig. 3D).

Although MCP-1 primarily binds to CCR2, recent evidence suggests that MCP-1 has non-CCR2-mediated effects in SMC including binding of the CCR4 receptor (32,33). Therefore, we intercrossed $Nf1^{+/-}$ and $CCR2^{-/-}$ mice to examine the role of CCR2 activation on $Nf1^{+/-}$ neointima formation and macrophage infiltration into the vascular wall. The common carotid arteries of

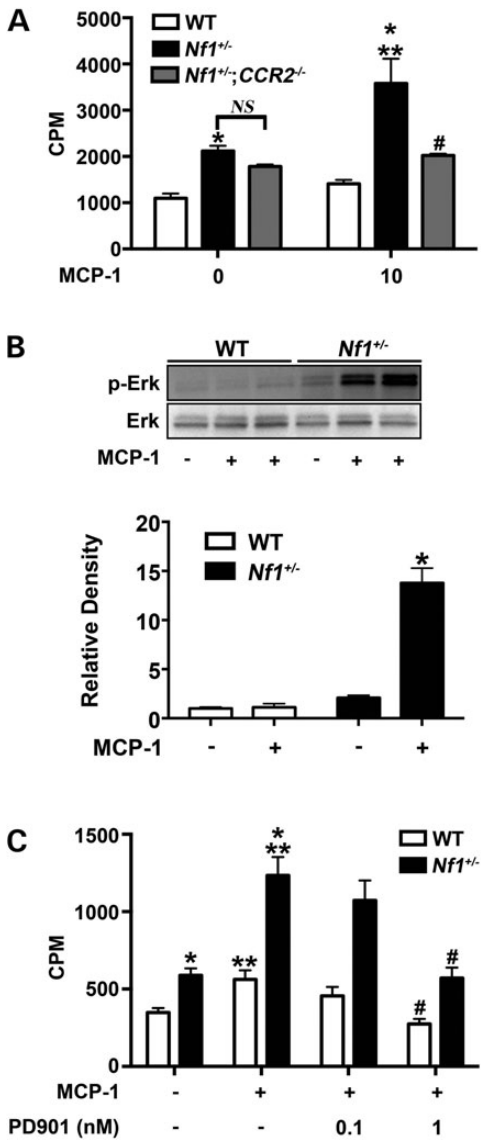

Figure 2. MCP-1 enhances $Nf1^{+/-}$ SMC proliferation via Erk activation. WT (white bars), $Nf1^{+/-}$ (black bars) and $Nf1^{+/-}$;$CCR2^{-/-}$ (gray bars) SMC proliferation in response to stimulation with MCP-1. (**A**) Data represent thymidine incorporation as mean cpm ± SEM, $n = 4$. *$P < 0.05$ for WT SMC versus $Nf1^{+/-}$ SMC at indicated concentrations of MCP-1. No statistical difference was observed between unstimulated $Nf1^{+/-}$ and $Nf1^{+/-}$;$CCR2^{-/-}$ SMC. **$P < 0.001$ for unstimulated $Nf1^{+/-}$ SMC versus MCP-1 stimulated $Nf1^{+/-}$ SMC. #$P < 0.001$ for MCP-1 stimulated $Nf1^{+/-}$ SMC versus MCP-1 stimulated $Nf1^{+/-}$;$CCR2^{-/-}$ SMC. (**B**) Representative western blot and quantitative densitometry of Erk phosphorylation in WT and $Nf1^{+/-}$ SMC treated with/without MCP-1 (10 ng/ml), $n = 3$. Quantitative densitometry ± SEM is reported as ratio for phosphorylated-Erk to total Erk density and corrected to unstimulated WT SMC. *$P < 0.0001$ for all conditions versus $Nf1^{+/-}$ SMC stimulated with MCP-1. (**C**) Data represent thymidine incorporation as mean cpm ± SEM, $n = 3$. *$P < 0.05$ for WT SMC versus $Nf1^{+/-}$ SMC at indicated concentrations of MCP-1. **$P < 0.01$ for unstimulated $Nf1^{+/-}$ and WT SMC versus MCP-1 stimulated $Nf1^{+/-}$ and WT SMC. #$P < 0.01$ for MCP-1 stimulated $Nf1^{+/-}$ and WT SMC versus MCP-1 stimulated $Nf1^{+/-}$ and WT SMC in the presence of PD0325901 at indicated concentration.

WT, $CCR2^{-/-}$, $Nf1^{+/-}$ and $Nf1^{+/-}$; $CCR2^{-/-}$ mice were ligated and analyzed for neointima formation after a 28-day recovery period. Similar to our previous experiment, $Nf1^{+/-}$ mice form a large neointima when compared with WT mice. Homozygous deletion of *CCR2* in $Nf1^{+/-}$ mice completely abrogated neointima formation and quantitative analysis of arterial cross sections demonstrated that arterial remodeling and neointima size were similar to WT arteries (Fig. 4A–C). Consistent with these observations, Mac-3+ macrophage infiltration into the neointima was reduced in $Nf1^{+/-}$;$CCR2^{-/-}$ mice when compared with $Nf1^{+/-}$ mice (12.2 ± 2.9% versus 21.53 ± 3.5% of total cell number, $P < 0.05$; Fig. 4D). Thus, MCP-1 and CCR2 expression are critical for neointima formation in $Nf1^{+/-}$ mice.

Pharmacologic inhibition of CCR2 inhibits $Nf1^{+/-}$ neointima formation

Pharmacologic inhibition of CCR2 could provide an attractive therapeutic target for NF1 patients with evidence of vasculopathy. Therefore, we utilized a specific CCR2 antagonist (INCB3284) for preclinical testing in our murine model system of NF1 arterial stenosis (34). $Nf1^{+/-}$ and WT mice were administered INCB3284 or vehicle via intraperitoneal injection immediately after arterial injury and once daily for 10 days as rescue therapy. This regimen was selected based on published pharmacokinetics for INCB3284 and specific targeting of macrophages during the early phase of arterial remodeling (34). In response to carotid artery ligation, vehicle-treated $Nf1^{+/-}$ mice developed significant intimal hyperplasia, whereas WT mice developed a more modest neointima, which was grossly and quantitatively similar to our previous observations (Fig. 5A–C). Daily administration of INCB 3284 as a rescue therapy significantly reduced neointima formation in $Nf1^{+/-}$ mice when compared with vehicle-treated $Nf1^{+/-}$ mice (Fig. 5B and C). While there was a trend toward reduction of neointima area and I/M ratio in WT mice treated with INCB 3284 when compared with WT mice receiving PBS treatment, statistical significance was not achieved ($P = 0.11$ and 0.5, respectively). Mac-3+ macrophage content was also reduced in $Nf1^{+/-}$ mice treated with INCB 3284 when compared with $Nf1^{+/-}$ mice receiving PBS treatment (16.3 ± 3.2% versus 24.12 ± 3.1% of total cell number, $P < 0.05$; Fig. 5D). Weight gain was similar between INCB 3284 and vehicle treatment groups, and no toxicities were observed on autopsy and inspection of visceral organs. These are the first data to suggest that a competitive CCR2 antagonist may be a viable therapeutic intervention for NF1 patients with evidence of cardiovascular disease.

Discussion

To date, therapeutic interventions for NF1 patients with cardiovascular disease have been limited, and tailored therapies directed at neurofibromin deficiency or its downstream targets are non-existent. Multiple extracellular signaling inputs converge on canonical Ras to maintain normal cell turnover, which limits the long-term use of Ras kinase inhibitors in patients with NF1 cardiovascular disease. Thus, a complete understanding of the pathogenesis of arterial disease in NF1 patients is imperative to inform novel therapeutic approaches leading to disease-specific clinical trials. Along this line of reasoning, emerging evidence suggests that NF1 patients experience chronic inflammation including increased cytokine production and frequency of circulating proinflammatory $CD14^{+}CD16^{+}$ monocytes in their peripheral blood (13). These findings are supported by our recent observation that $Nf1^{+/-}$ mice have increased circulating $Ly6C^{hi}$ monocytes, which closely resemble human proinflammatory intermediate monocytes (16). Increased cell surface expression of CCR2 is characteristic of $Ly6C^{hi}$ monocytes and enables these primitive myeloid cells to emigrate from the bone marrow and home to sites of inflammation where they differentiate into

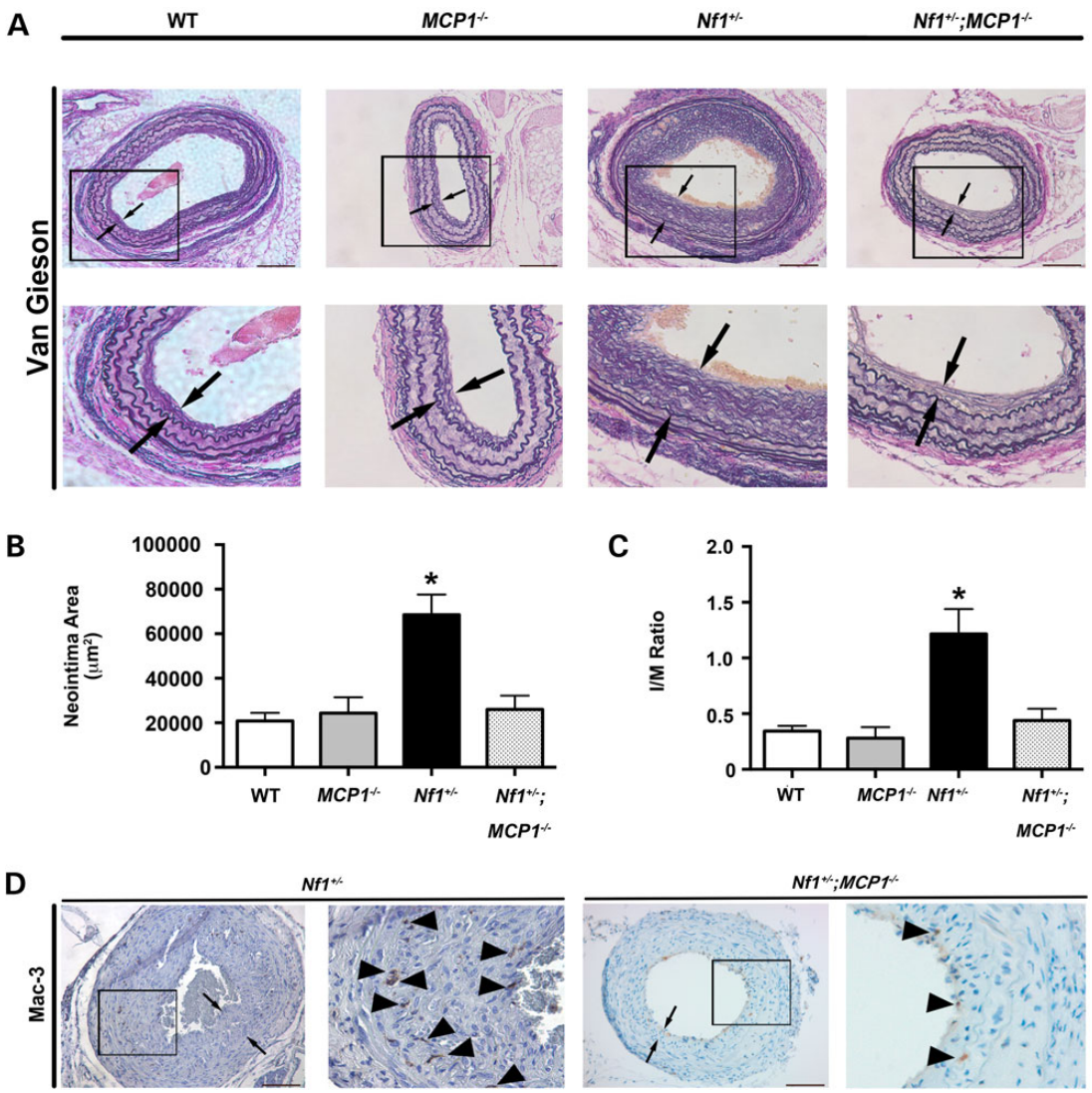

Figure 3. Genetic deletion of MCP-1 inhibits $Nf1^{+/-}$ neointima formation. Representative photomicrographs (**A**) and quantification of neointima area (**B** and **C**) of injured carotid arteries from WT, $MCP1^{-/-}$, $Nf1^{+/-}$ and $Nf1^{+/-}$;$MCP1^{-/-}$ mice. (A) Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified below. Scale bars: 100 μm. (B and C) Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from WT, $MCP1^{-/-}$, $Nf1^{+/-}$ and $Nf1^{+/-}$; $MCP1^{-/-}$ mice. Data represent mean neointima area or I/M ratio ± SEM, $n = 10–12$. *$P < 0.01$ for WT, $MCP1^{-/-}$ and $Nf1^{+/-}$;$MCP1^{-/-}$ mice versus $Nf1^{+/-}$ mice. (**D**) Representative photomicrographs of Mac-3 staining in injured carotid arteries from $Nf1^{+/-}$ and $Nf1^{+/-}$;$MCP1^{-/-}$ mice. Black arrows indicate neointima boundaries. Black box identifies area of injured artery that is magnified in the right column. Black arrowheads represent positive macrophage (anti-Mac3) staining. Scale bars: 100 μm.

macrophages and secrete growth factors, reactive oxygen species and cytokines (35). Neurofibromin appears to play a central role in their derivation and mobilization from the bone marrow. Similar to $Nf1^{+/-}$ mice, mice harboring a lineage-restricted deletion of a single *Nf1* gene copy in myeloid cells have increased Ly6-C^{hi}CCR2+ monocytes in the peripheral blood (15). Interestingly, genetic deletion of both *Nf1* gene copies resulted in a 4-fold increase in circulating Ly6C^{hi}CCR2+ monocyte frequency, which strongly suggests that neurofibromin directly regulates monocyte mobilization and inflammation via a cell autonomous and gene-dosage-dependent mechanism (15).

Not surprisingly, monocytes and macrophages appear to play a central role in the pathogenesis of NF1-related arterial stenosis. Experimental ligation of the common carotid artery in mice harboring a myeloid-specific deletion of *Nf1* results in a robust neointima that is identical to $Nf1^{+/-}$ mice and NF1 patients. Deletion of both *Nf1* alleles in myeloid cells resulted in a near-total occlusion of the carotid artery after injury. Thus, neointima formation in *Nf1*-mutant mice is directly regulated by neurofibromin expression in myeloid cells via a gene-dosage-dependent mechanism. Based on the observation that neointima formation and CCR2+, inflammatory monocyte frequency is directly regulated by myeloid cell-specific mutations in the *Nf1* gene, we utilized MCP-1 and CCR2 knockout mice to specifically interrogate the role of neurofibromin in regulating CCR2-dependent monocyte mobilization and homing during cardiovascular remodeling. Here we show that loss of MCP-1 or CCR2 expression prevents macrophage infiltration into the arterial wall and abolishes neointima formation in $Nf1^{+/-}$ mice. The recruitment of bone marrow monocytes appears to be strongly linked to the presence and activation of CCR2. CCR2-deficient mice have a dramatic reduction in mature monocyte frequency in the peripheral blood, whereas the bone marrow appears to be enriched with primitive and precursor myeloid cells indicating that CCR2 participates in

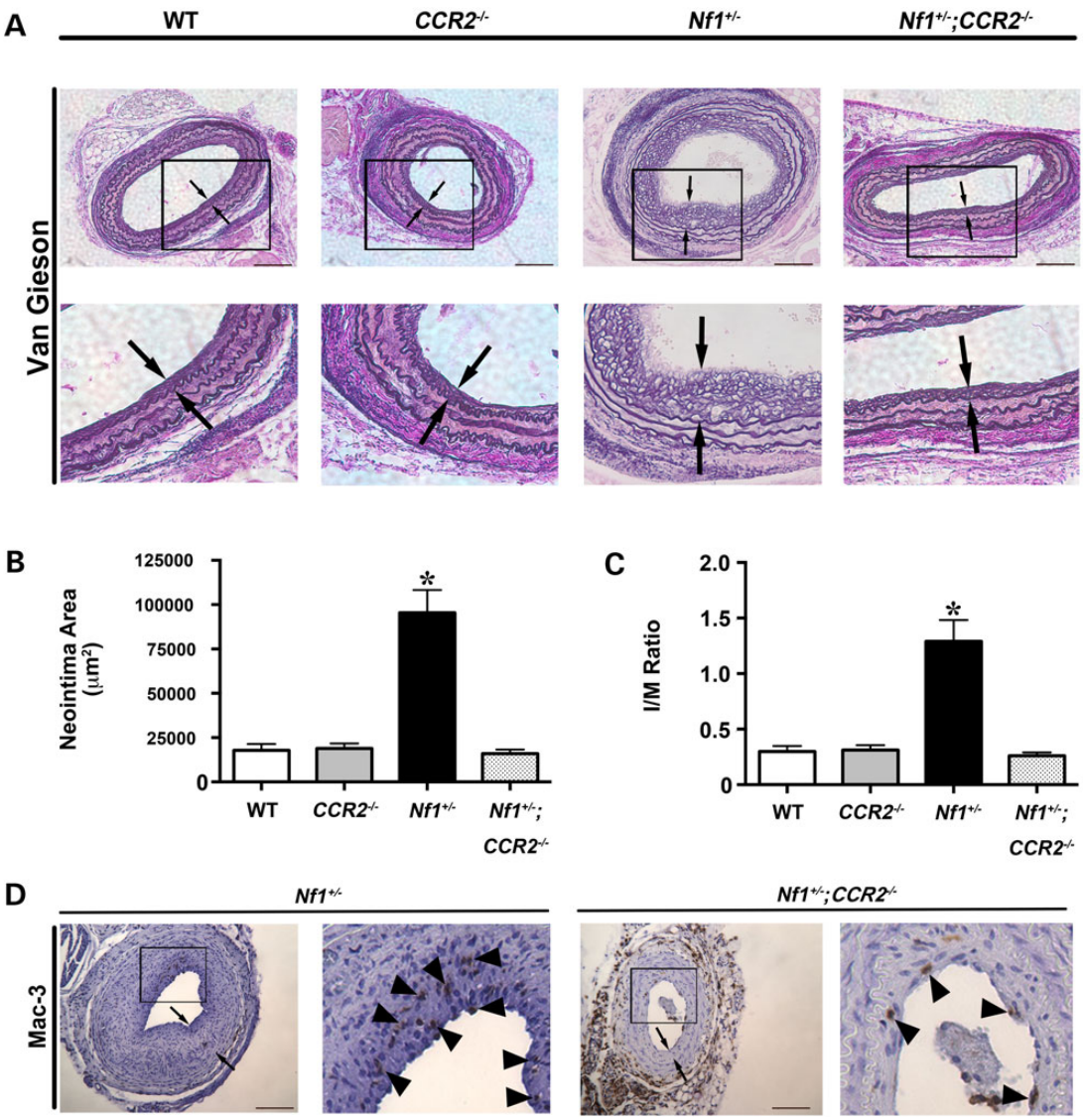

Figure 4. Genetic deletion of *CCR2* inhibits $Nf1^{+/-}$ neointima formation. Representative photomicrographs (**A**) and quantification of neointima area (**B** and **C**) of injured carotid arteries from WT, $CCR2^{-/-}$, $Nf1^{+/-}$ and $Nf1^{+/-}$;$CCR2^{-/-}$ mice. (A) Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified below. Scale bars: 100 μm. (B and C) Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from WT, $CCR2^{-/-}$, $Nf1^{+/-}$ and $Nf1^{+/-}$;$CCR2^{-/-}$ mice. Data represent mean neointima area or I/M ratio ± SEM, $n = 10–12$. *$P < 0.005$ for WT, $CCR2^{-/-}$ and $Nf1^{+/-}$;$CCR2^{-/-}$ mice versus $Nf1^{+/-}$ mice. (**D**) Representative photomicrographs of Mac-3 staining in injured carotid arteries from $Nf1^{+/-}$ and $Nf1^{+/-}$;$CCR2^{-/-}$ mice. Black arrows indicate neointima boundaries. Black box identifies area of injured artery that is magnified in the right column. Black arrowheads represent positive macrophage (anti-Mac3) staining. Scale bars: 100 μm.

the mobilization of monocytes from the bone marrow (25,36,37). Monocyte differentiation in $CCR2^{-/-}$ mice remains intact; however, mature monocytes fail to accumulate in the spleen and respond to inflammatory stimuli (36). Surprisingly, *CCR2* deletion failed to reduce neointima formation in our experimental model system. The demonstrated reduction in neointima formation in CCR2-deficient mice has largely been demonstrated in compound mutant mice (i.e. $CCR2^{-/-}$;$ApoE^{-/-}$) and may rely on a hyperlipidemic background to mediate its effects (38,39). Further, C57Bl/6 mice are highly resistant to arterial remodeling in multiple animal models, which is consistent with our experimental results and previous reports (12,13,15,27,40).

Although MCP-1 is the principal ligand for CCR2, other monocyte chemotactic proteins have affinity for CCR2 and have a role in monocyte function, SMC proliferation and arterial remodeling (25,41,42). Thus, complimentary studies in $Nf1^{+/-}$ mice lacking MCP-1 expression were critical to link the increased sensitivity of neurofibromin-deficient monocytes/macrophages to MCP-1 and the mobilization of CCR2+ proinflammatory monocytes in *Nf1*-mutant mice. The lack of neointima formation in $Nf1^{+/-}$;$MCP1^{-/-}$ mice mirrors our findings in CCR2-deficient $Nf1^{+/-}$ mice and provides strong genetic evidence that this signaling axis is necessary for $Nf1^{+/-}$ neointima formation. The underlying mechanisms of this interaction remain unclear; however, we show that MCP-1 preferentially activates Erk signaling in $Nf1^{+/-}$ SMC and multiple Ras-dependent kinases in $Nf1^{+/-}$ monocytes, including Akt. Interestingly, Erk and Akt, the primary downstream targets of Ras, activate several transcription factors including SP-1, c-Jun and AP-1, which control the expression of MCP-1 and other cytokines (43–46). In turn, expression of chemokines such as MCP-1 largely controls leukocyte and SMC function via RTK-mediated Ras activation. This may contribute to a positive feedback loop where

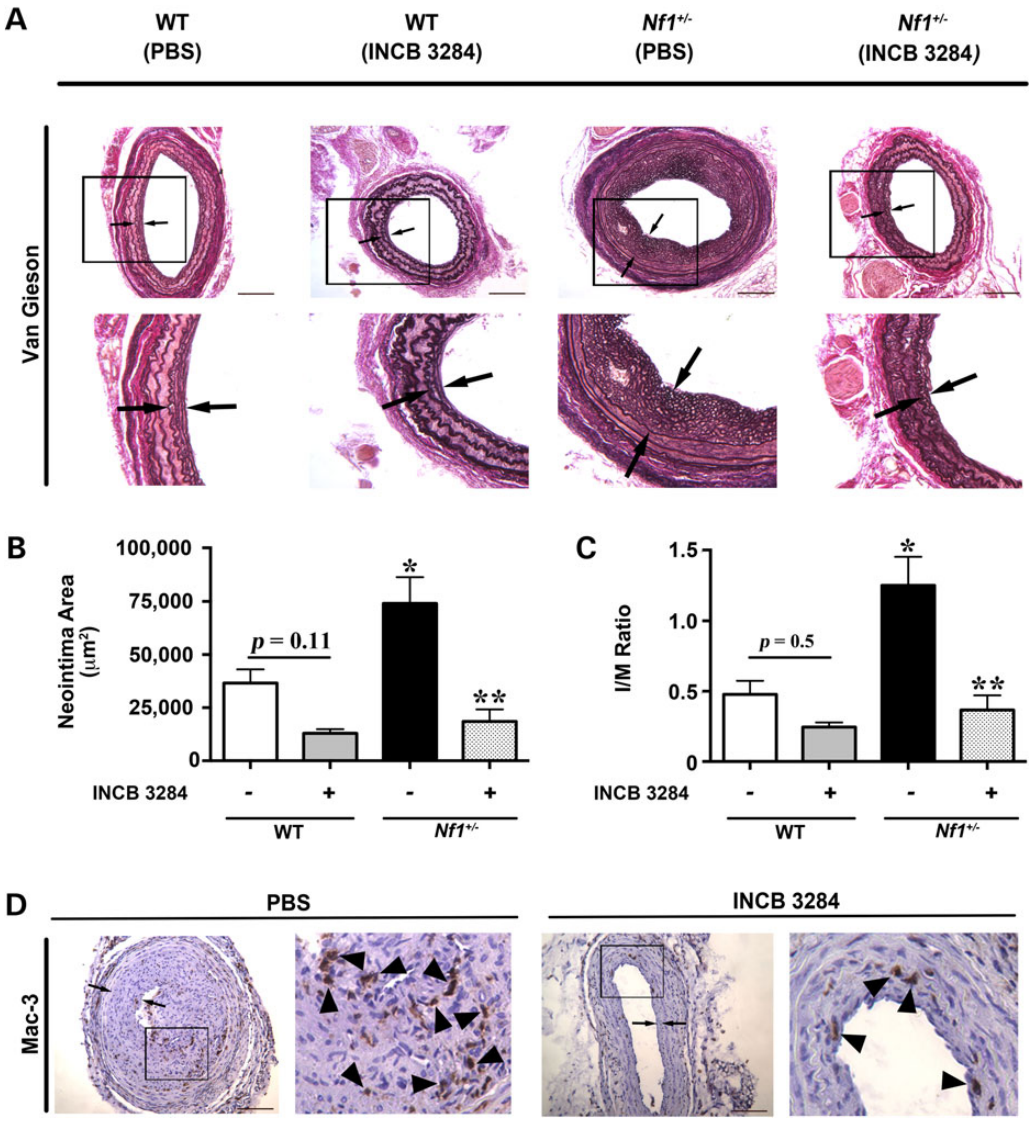

Figure 5. CCR2 antagonist reduces neointima formation in $Nf1^{+/-}$ mice. Representative photomicrographs (**A**) and quantification of neointima area (**B** and **C**) of injured carotid arteries from WT and $Nf1^{+/-}$ mice treated with INCB 3284 (15 mg/kg/day) or PBS. (A) Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified below. Scale bars: 100 μm. (B and C) Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from WT and $Nf1^{+/-}$ mice treated with INCB 3284 or PBS. Data represent mean neointima area or I/M ratio ± SEM, $n = 6–8$. *$P < 0.01$ for WT mice with PBS treatment versus $Nf1^{+/-}$ mice with PBS treatment. **$P < 0.001$ for $Nf1^{+/-}$ mice with PBS treatment versus $Nf1^{+/-}$ mice with INCB 3284 treatment. No statistically significant difference was observed between WT mice with PBS treatment and WT mice with INCB 3284 treatment. Experiments were performed in triplicate. (**D**) Representative photomicrographs of Mac-3 staining in injured carotid arteries from $Nf1^{+/-}$ mice with PBS treatment versus $Nf1^{+/-}$ mice with INCB 3284 treatment. Black arrows indicate neointima boundaries. Black box identifies area of injured artery that is magnified in the right column. Black arrowheads represent positive macrophage (anti-Mac3) staining. Scale bars: 100 μm.

cytokines can propagate their own production, which may be amplified in the setting of Ras deregulation (47–49). Our observation that neurofibromin-deficient macrophages have increased production and secretion of MCP-1, which may potentiate their own function, is congruent with this line of investigation. For example, mTOR signaling is proximally regulated by neurofibromin and loss of neurofibromin increases Akt activity in response to MCP-1 in murine monocytes. Recent studies in human and murine monocytes have revealed that MCP-1 production is directly regulated by mTOR activation and that inhibition of mTOR signaling results in decreased MCP-1 expression and secretion via an NF-κB-dependent mechanism. Interestingly genetic deletion of tuberous sclerosis complex 2 amplifies mTOR signaling and also increases MCP-1 expression, which suggests that this pathway directly regulates MCP-1 production. Therefore, it is plausible that decreased expression of neurofibromin may enhance downstream signaling networks that increase MCP-1 expression to provide a greater local concentration of MCP-1 to activate CCR2 on circulating monocytes/macrophages and vascular wall cells. While long-term use of an mTOR inhibitor for the prevention or treatment of NF1 vasculopathy is unlikely, interrogation of this pathway may provide substantial mechanistic insights into the regulation of monocyte-specific chemokines and their function in the setting of neurofibromin deficiency. Ongoing studies in our laboratory are focused on understanding how Ras signaling controls the transcription and/or activity of MCP-1.

Targeted therapies directed against either MCP-1 have yielded promising results in preclinical animal models, but have largely

proven to lack efficacy in human clinical trials (50–55). Bindarit, an indazolic derivative that binds to the promoter of the MCP-1 gene and limits MCP-1 expression, inhibits human and murine SMC proliferation and de-differentiation as well as neointima formation in several animal models (28,55,56). However, the use of a monoclonal antibody directed against MCP-1 in persons with rheumatoid arthritis did not improve clinical symptoms and demonstrated a dose-responsive increase in serum MCP-1 concentrations, which suggests that clearance of the antibody complex is limited (57). Antibodies directed against MCP-1 in the treatment of cardiovascular disease have not been proposed, though bindarit is presently under investigation for the treatment of diabetic nephropathy. MCP-1 may prove to be a difficult target for the treatment of cardiovascular disease because it is expressed and secreted by multiple cell types, including EC, SMC, leukocytes and perivascular adipocytes, and demonstrates affinity for other receptors including CCR4 (32,33). Further, MCP-1 secretion provides important feedback between perivascular cells (i.e. adipocytes) and remote tissues, including skeletal muscle and hepatocytes (58,59), which makes sequestering MCP-1 activity clinically difficult.

Local inhibition of CCR2, on the other hand, is an attractive therapeutic target for the treatment of NF1 vasculopathy because its expression and ligand-binding affinity is relatively limited. CCR2 antagonists have been studied in multiple clinical trials for the treatment of cardiovascular disease, autoimmunity, chronic inflammation, diabetes and malignancy (60–62). In particular, CCR2 antagonism may prove efficacious in the treatment of multiple manifestations of NF1, because acceleration of Ras activity in myeloid progenitor cells leads to dysfunctional differentiation and increased sensitivity to cytokines and growth factors (1,63–65). Our genetic and pharmacologic data support our hypothesis that MCP-1/CCR2 activation is highly regulated by neurofibromin and may represent a viable therapeutic target for NF1 patients with cardiovascular disease. Further studies directed at understanding how neurofibromin and/or Ras activation control the expression of MCP-1 and facilitate the mobilization of CCR2+ monocytes from the bone marrow are critical for future translational work and human studies of NF1 vasculopathy.

Materials and Methods

Animals

Protocols were approved by Laboratory Animal Services at Augusta University and Indiana University. *Nf1*$^{+/-}$ mice were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA) and backcrossed 13 generations into the C57BL/6J strain. *MCP-1* (4434) and *CCR2* (4999) knockout mice were purchased from The Jackson Laboratory and maintained on C57BL/6 strain. *Nf1*$^{+/-}$ mice were intercrossed with *MCP1* and *CCR2* knockout mice to produce *Nf1*$^{+/-}$;*MCP1*$^{-/-}$ and *Nf1*$^{+/-}$;*CCR2*$^{-/-}$ mice. Male mice (12–15 weeks of age) were used for experiments to limit the confounding effects of circulating hormones.

Carotid artery ligation

Carotid artery injury was induced by ligation of the right common carotid artery as described (15). Briefly, mice were anesthetized by inhalation of an isoflurane (2%)/oxygen (98%) mixture. Under a dissecting scope, the right carotid artery was exposed through a midline neck incision and ligated proximal to the bifurcation using a 6–0 silk suture. The contralateral carotid artery was sham ligated as a control. Mice were administered 15 μg of buprenorphine (ip) following the procedure and recovered for 28 days. In some experiments, *Nf1*$^{+/-}$ and WT mice were administered 15 mg/kg INCB3284 (Caymen Chemical, IC_{50} 3.7 nM and $t_{½}$ 15 h) or vehicle via IP injection immediately after arterial injury and continued once daily for 10 days.

Morphometric analysis

Van Gieson-stained arterial cross sections 400, 800 and 1200 μm proximal to the ligation were analyzed for neointima formation using ImageJ (NIH, Bethesda, MD). Lumen area, area inside the internal elastic lamina (IEL), and area inside the external elastic lamina (EEL) were measured for each cross section. To account for potential thrombus formation, arteries containing significant thrombus (>50% lumen occlusion) at 400 μm proximal to the ligation were excluded from analysis. The number of excluded arteries was not different between experimental groups. Representative photomicrographs for each figure are taken from arterial cross sections between 600 and 1200 μm proximal to the bifurcation. Intima area was calculated by subtracting the lumen area from the IEL area, and the media area was calculated by subtracting the IEL area from the EEL area. I/M ratio was calculated as intima area divided by media area.

Histopathology and immunohistochemistry

For immunohistochemistry, serial sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol following antigen retrieval in Antigen Unmasking Solution (Vector Laboratories) at 95°C. Sections were blocked with Protein Block (Dako) for 1 h and were incubated with anti-Mac3 (1:50; BD Biosciences) primary antibodies. Sections were incubated with a biotinylated secondary antibody and visualized by 3,3′-diaminobenidine and counterstained with hematoxylin. Sections were examined, and images of sections were collected using a Zeiss Axioskop microscope (Carl Zeiss) with a 20× or 40× CP-ACHROMAT/0.12NA objective. Images were acquired using a SPOT RT color camera (Diagnostic Instruments). To quantify the number of macrophages within the neointima of each experimental group, Mac-3+ cells and SMCs were counted in three random 40× images by a blinded observer. To correct for a reduction in cell volume within the neointima, a ratio of Mac-3+ cells and SMC was calculated and analyzed for each mouse.

Isolation of bone-marrow-derived macrophages and characterization

Bone-marrow-derived macrophage isolation and characterization was performed as described (13). Proliferation was assessed by incorporation of radioactive thymidine in WT and *Nf1*$^{+/-}$ BM-derived macrophages. Briefly, WT and *Nf1*$^{+/-}$ macrophages (5×10^4 cells) were serum-starved for 12–18 h and placed in a 96-well plate in 200 μl starvation media in either the absence or presence of MCP-1 (10 ng/ml). Cells were cultured for 24 h and subsequently pulsed with 1.0 μCi (0.037 MBq) [^{3}H] thymidine for 6 h. Cells were harvested using a cell harvester and thymidine incorporation was determined as counts per minute (cpm).

For macrophage migration, the bottom of Transwell filters (8-μm pore filter; Costar) were coated with 20 μg/ml fibronectin CH296 peptide for 2 h at 37°C and rinsed twice with PBS containing 2% BSA. WT and *Nf1*$^{+/-}$ macrophages (2.5×10^5 cells) were placed in the upper chamber of the transwell and allowed to migrate toward the bottom of the transwell containing indicated

concentration of MCP-1. After 24 h, non-migrated cells in the upper chamber were removed with a cotton swab and migrated cells that attached to the bottom surface of the membrane were stained with 0.1% crystal violet dissolved in 0.1 M borate, pH 9.0 and 2% ethanol for 5 min at room temperature. The number of migrated cells was determined in five random fields with an inverted microscope using a 20 × objective lens. All experiments were performed in triplicate.

Smooth muscle cell isolation and proliferation

SMC isolation and proliferation assays were performed as described (14). SMCs were obtained by outgrowth from explants of WT, $Nf1^{+/-}$ and $Nf1^{+/-}$;$CCR2^{-/-}$ thoracic aortas. SMCs were cultured in DMEM supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin in a 37°C, 5% CO_2-humidified incubator. For cell proliferation, SMC (5000 cells/cm^2) were placed in a 96-well plate and deprived of growth factors for 12–18 h. Quiescent SMC were stimulated with MCP-1 (10 ng/ml) for 24 h and pulse-labeled with 1 µCi/ml of [^{3}H] thymidine for 6 h. Beta emission was measured and reported as cpm. In some experiments, SMCs were incubated with indicated concentrations of PD0325901 (Erk inhibitor). All experiments were performed in triplicate.

Statistical analysis

All values are presented as mean or percent ± SEM. Cell proliferation and migration were analyzed by two-way ANOVA with Tukey's post hoc test for multiple comparisons. All experiments were performed in triplicate. MCP-1 concentration was analyzed by Student's *t*-test. Intima area and I/M ratio analysis was assessed by one-way ANOVA with Tukey's post hoc test for multiple comparisons. Murine experiments utilizing INCB 3284 were analyzed using two-way ANOVA with Tukey's post hoc test for multiple comparisons. Percent Mac-3+ cells was analyzed by Student's *t*-test. Analysis was performed using GraphPad Prism version 5.0d. $P < 0.05$ were considered significant.

Conflict of Interest statement. None declared.

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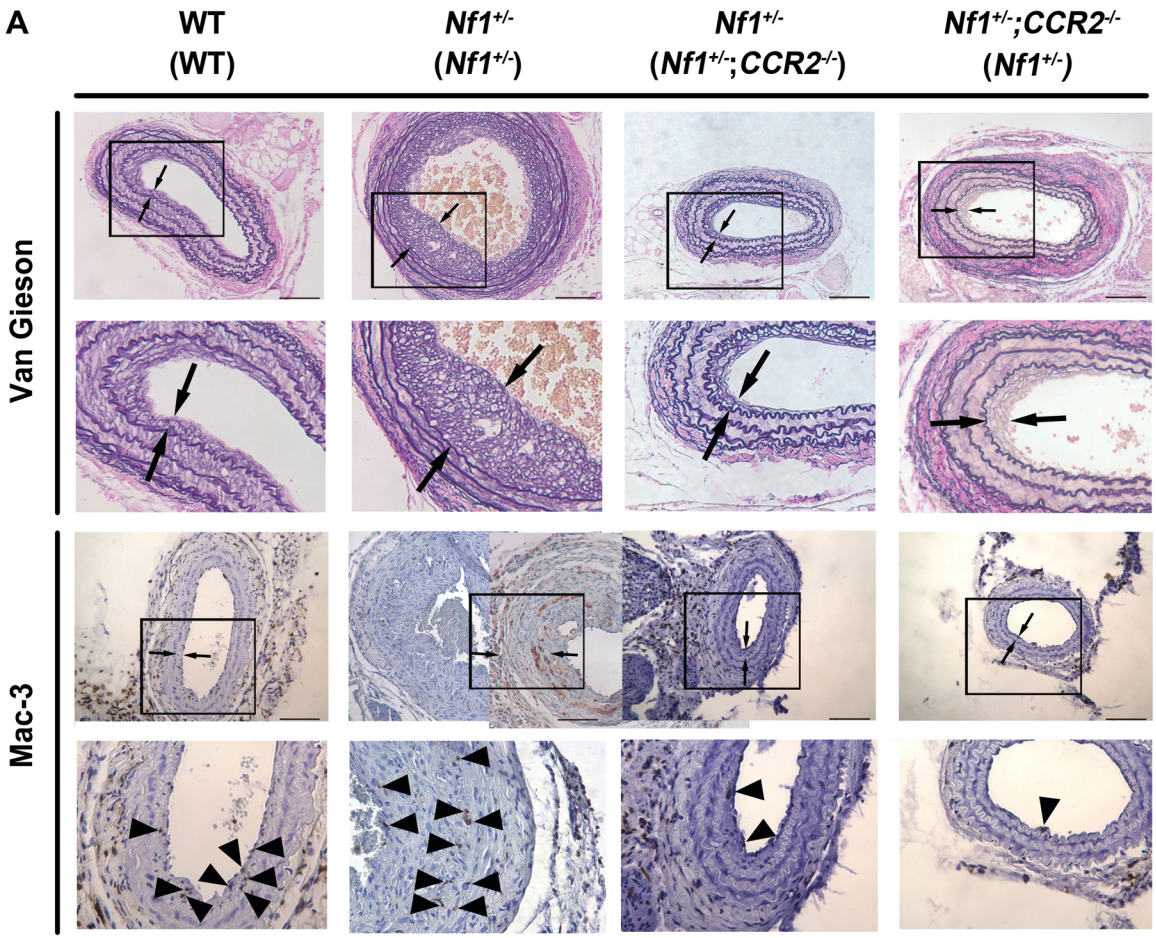
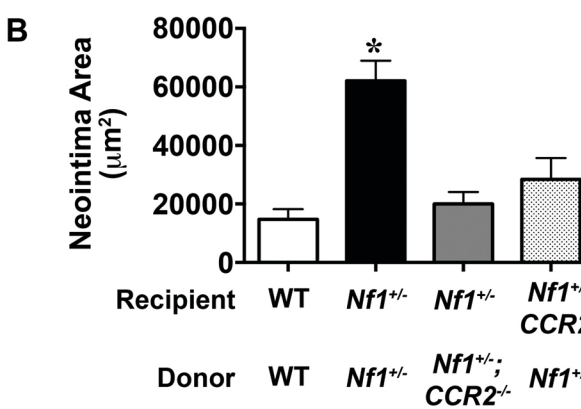
Figure 1


A
WT
(WT)
Nf1+/-
(Nf1+/-)
Nf1+/-
(Nf1+/-;CCR2-/-)
Nf1+/-;CCR2-/-
(Nf1+/-)
Van Gieson
Mac-3


B
Neointima Area
(μm2)
80000
60000
40000
20000
0
*
Recipient
WT
Nf1+/-
Nf1+/-
Nf1+/-;
CCR2-/-
Donor
WT
Nf1+/-
Nf1+/-;
CCR2-/-
Nf1+/-

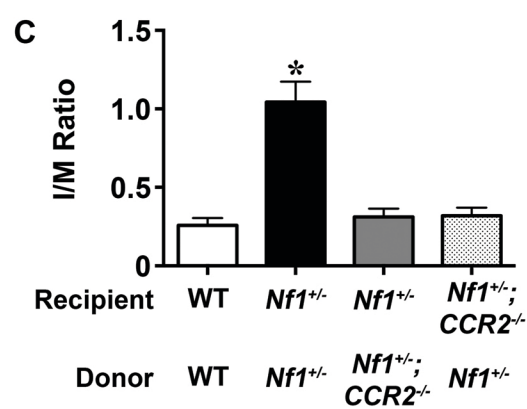

C
I/M Ratio
1.5
1.0
0.5
0
*
Recipient
WT
Nf1+/-
Nf1+/-
Nf1+/-;
CCR2-/-
Donor
WT
Nf1+/-
Nf1+/-;
CCR2-/-
Nf1+/-

Figure 2

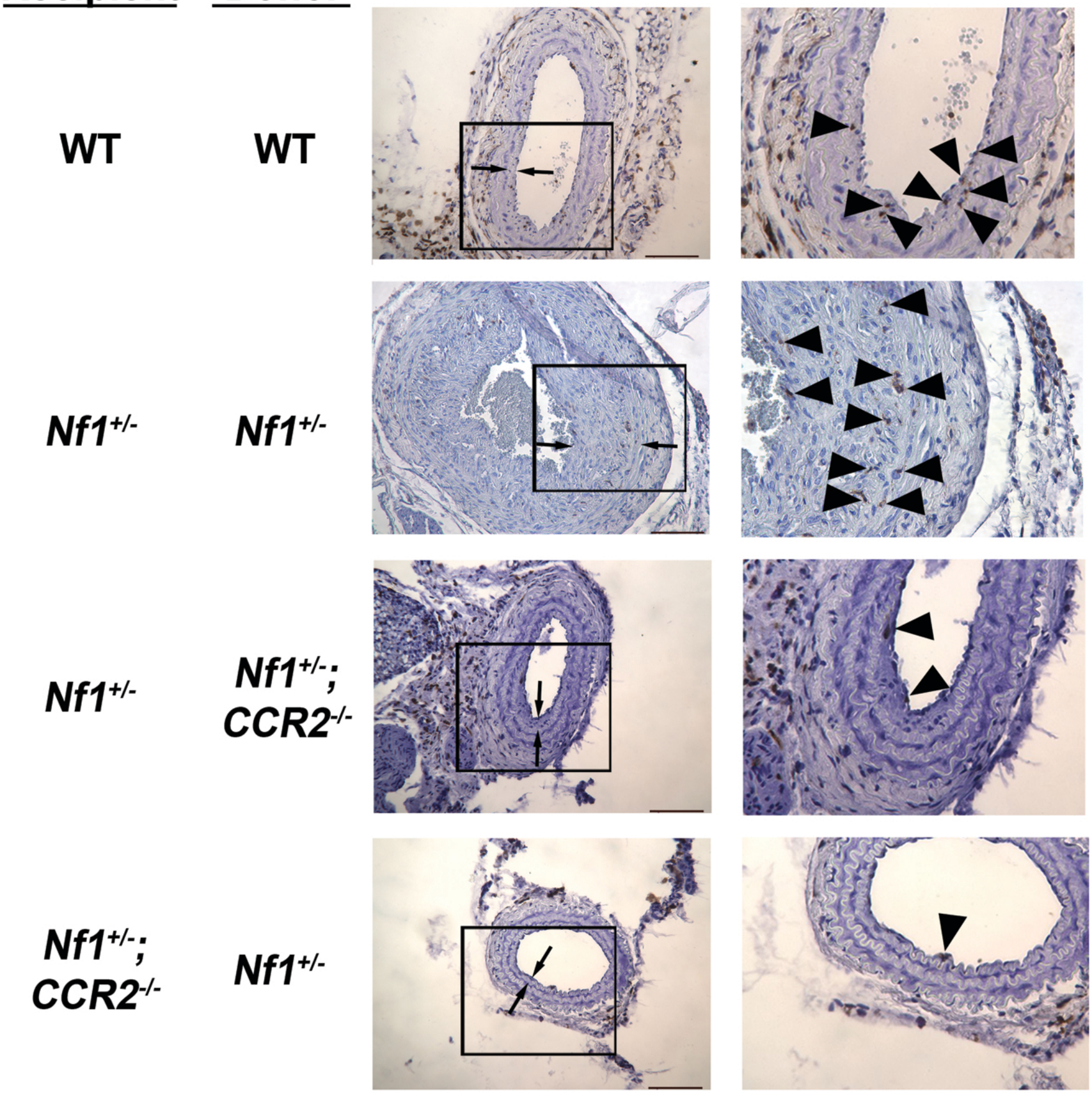
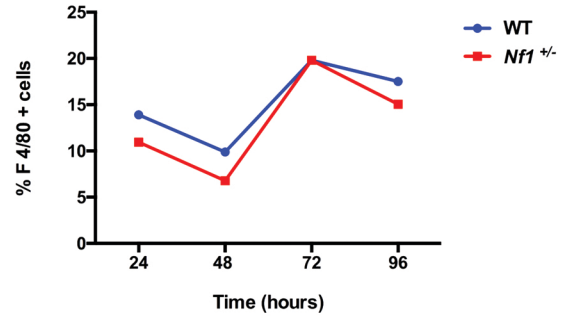
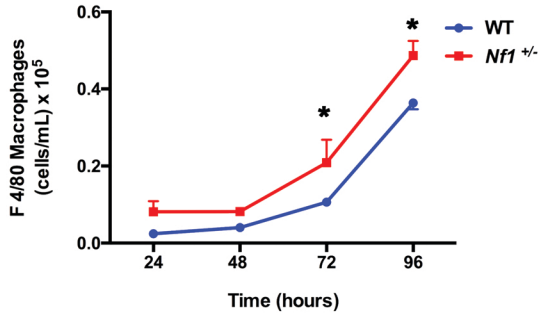
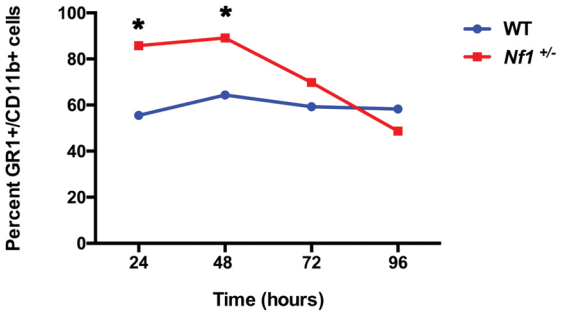


Figure 3

A


% F 4/80 + cells
25
20
15
10
5
0
24
48
72
96
Time (hours)
WT
Nf1 +/-


F 4/80 Macrophages (cells/mL) x 10^5
0.6
0.4
0.2
0.0
*
*
24
48
72
96
Time (hours)
WT
Nf1 +/-


Percent GR1+/CD11b+ cells
100
80
60
40
20
0
*
*
24
48
72
96
Time (hours)
WT
Nf1 +/-

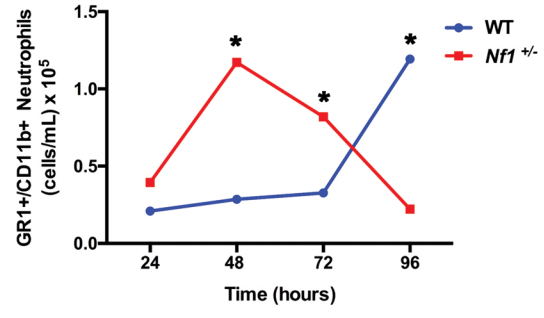

GR1+/CD11b+ Neutrophils (cells/mL) x 10^5
1.5
1.0
0.5
0.0
*
*
*
24
48
72
96
Time (hours)
WT
Nf1 +/-

Figure 4

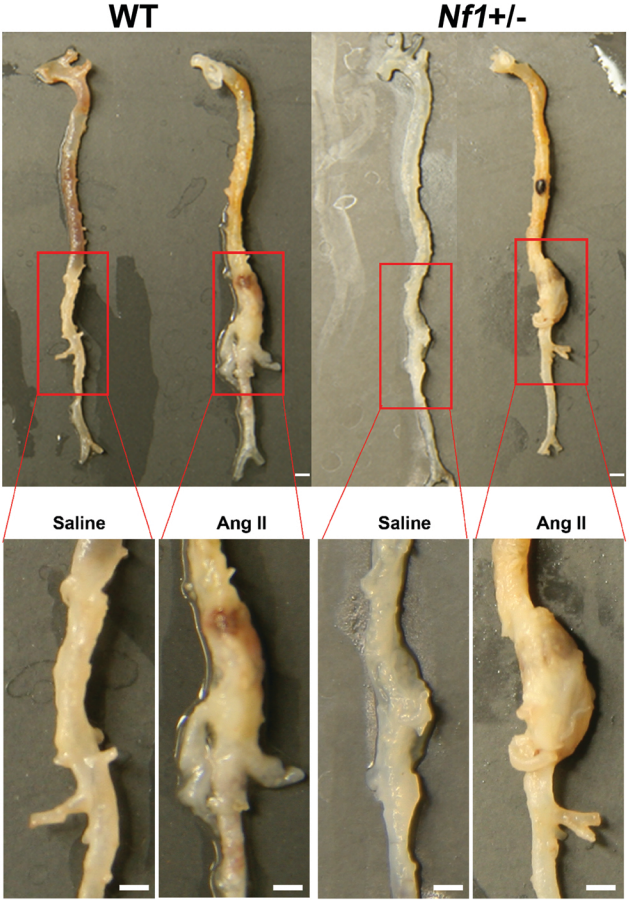
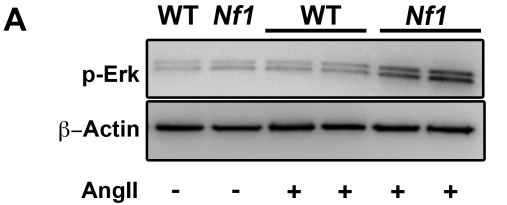
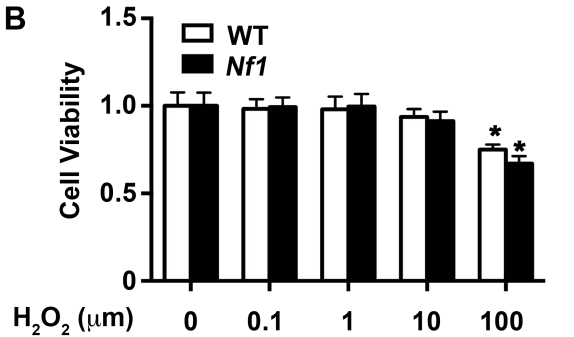
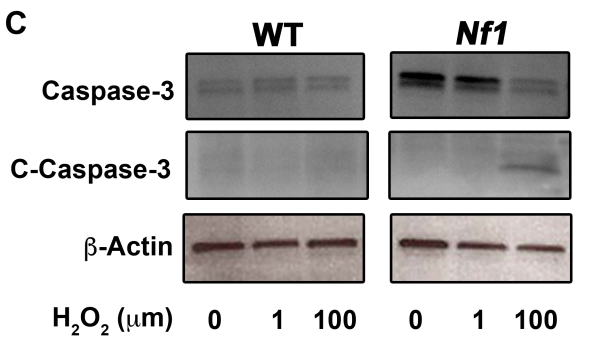


Figure 5


A
WT
Nf1
WT
Nf1
p-Erk
β–Actin
AngII - - + + + +


B
1.5
1.0
0.5
0
WT
Nf1
Cell Viability
*
*
H2O2 (μm)
0
0.1
1
10
100


C
WT
Nf1
Caspase-3
C-Caspase-3
β-Actin
H2O2 (μm)
0
1
100
0
1
100

CURRICULUM VITAE

NAME: Brian Kevin Stansfield, M.D.

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1120 15th St, BIW 6033
Augusta, GA 30912
P: 706.721.5059
F: 706.721.7531
bstansfield@augusta.edu

EDUCATION:

UNDERGRADUATE:

1996-2000	Georgia Southern University, Statesboro, GA	B.S. Chemistry

GRADUATE:

2000-2004	Medical College of Georgia, Augusta, GA	M.D.

POSTDOCTORAL:

2004-2007	Resident Physician in Pediatrics	Medical College of Georgia
2009-2011	Fellow in Neonatal-Perinatal Medicine	Indiana University School of Medicine
2011-2013	NIH Fellow in Pediatric Scientist Development Program	Indiana University School of Medicine

ACADEMIC APPOINTMENTS:

2007-2009	Instructor of Clinical Pediatrics, Medical College of Georgia
2013-present	Assistant Professor of Pediatrics, Augusta University
2015-present	College of Graduate Studies, Augusta University

HOSPITAL APPOINTMENTS:

2007-2009	Attending Physician, University Hospital Emergency Department, Augusta, GA
2011-2013	Attending Physician, Indiana University Health North Hospitals, Indianapolis, IN
2013-present	Attending Physician, Augusta University Hospital, Augusta, GA

SPECIALTY BOARD STATUS:

2007	American Board of Pediatrics – General Pediatrics
2014	American Board of Pediatrics – Neonatal/Perinatal Medicine

LICENSURE AND CERTIFICATION:

2007-2011	South Carolina Medical License
2009-2013	Indiana Medical License
2007-present	Georgia Medical License

PROFESSIONAL ORGANIZATIONS:

2004-present	Member, American Academy of Pediatrics
2007-present	Member, American Medical Association
2009-present	Member, AAP section on Perinatal Pediatrics
2009-2013	Member, AAP section on Medical Student, Resident and Fellowship Trainees
2009-2013	Member, Indiana Medical Society
2010-present	Member, American Society of Hematology
2010-present	Member, American Heart Association
2014-present	Member, Southern Society for Pediatric Research
2016-present	Member, Society for Pediatric Research

HONORS AND AWARDS:

2000	Magna Cum Laude, Georgia Southern University
2000	American Chemical Society Undergraduate Research Award
2007	William P. Kanto Resident Research Award, Medical College of Georgia
2010	Red Shoes Award for compassionate care at Riley Hospital for Children
2011	Pediatric Scientist Development Program Award
2012	Riley Scholar's Day Award
2012	Jack Metcoff Award for Outstanding Fellow Presentation by MWSPR
2013	NIH Loan Repayment Award
2015	"Caught in the Act of Great Teaching", Augusta University
2015	Basic Science Young Investigator Award by SSPR
2015	Basic Science Poster Award, Children's Tumor Foundation
2015	NIH Loan Repayment Competitive Renewal
2015	MCG Exemplary Teaching Award, Augusta University
2016	Clinical Science Young Investigator Award by SSPR
2016	Jag20 Emerging Alumni Leader Award, Augusta University
2017	SSPR Young Faculty Award

TEACHING ASSIGNMENTS:

MEDICAL FELLOWS:

2013-2015	Pinkal Patel, Neonatology, Augusta University
2015-2018	Emily Masoumy, Neonatology, Augusta University
2017-2018	Dusit Adstamongkonkul, Pediatric Neurology, Augusta University

MEDICAL RESIDENTS:

2011	Hilary White, Indiana University S.O.M.
2015-2016	Meredith Johnston, Augusta University M.C.G.
2016-2018	Kate McCutcheon, Augusta University M.C.G.
2016-2018	Stephanie Komic, Augusta University M.C.G.

GRADUATE STUDENTS:

2016-2020	Rebekah Tritz, Augusta University

MEDICAL STUDENTS:

2012	Jacob Capito, Indiana University S.O.M.
2014	Christopher Walker, Augusta University M.C.G.
2016	Stephanie Ryals, Augusta University M.C.G.
2016-2018	Alexandra Sawyer, Augusta University M.C.G.
2017	Jenny Patel, Augusta University M.C.G.
2017	Folasade Aderibigbe, Augusta University M.C.G.

UNDERGRADUATE STUDENTS:

2010	James Wodoka, Case Western University (MD/PhD at Indiana University)
2015-2017	Sanah Aslam (B.S./M.D. program), Augusta University
2016-2017	Alexandra Cetatoiu, Augusta University

UNIVERSITY SERVICE:

2010-2012	Wells Center for Pediatric Research summer intern program, IU S.O.M.
2013-present	PEDS 5000, Junior core rotation in Pediatrics, AU
2013-present	PEDS 5001, Sub-internship in Neonatology, AU
2013-present	Neonatal Fellowship Lecture Series, AU
2013-present	Children's Summer Scholars Program, AU
2014-present	PEDS 5037, Advanced Pediatric Elective, AU
2014-present	Neonatology Fellowship Clinical Competency Committee, AU
2015-present	Dean's Research Committee of the MCG Faculty Senate, AU
2015-present	Pediatric Residency Program Evaluation Committee, AU
2015-present	Pediatric Resident Mentor, AU
2015-present	Pediatric Clinical Competency Committee, AU
2015-present	Pediatric Fellows 1st Year Curriculum, AU
2015-2016	LCME Site Visit Representative, AU
2016-present	MD/PhD Admissions Committee Member, AU

OTHER PROFESSIONAL ACTIVITIES:

INVITED LECTURES:

2013	"Understanding the Pathogenesis of Vascular Disease in Neurofibromatosis Type I Patients: Insights from Mouse Models" Perinatal Grand Rounds. University of Alabama at Birmingham
2016	"Zika Virus in the Continental United States" Family Medicine Grand Rounds.

Augusta University

2017 "The Lifelong Effects of Early Life" Perinatal Grand Rounds. Medical University of South Carollina

AD HOC REVIEW
BMJ Open
Cellular and Molecular Life Sciences
Cellular Physiology and Biochemistry
Circulation Research
International Journal of Obesity
Journal of Maternal-Fetal & Neonatal Medicine
Journal of Perinatology
Pediatric Research
PLOS One

GRANTS AND FELLOWSHIPS:

PRIOR SUPPORT:

"Myeloid Cells and NF1 Vasculopathy" Pediatric Scientist Development Program
NICHD-K12HD000850-27
07/01/11-06/30/14, Total Cost $206,000
B. Stansfield, Principal Investigator

Department of Pediatrics, Fellow Training Grant, Indiana University School of Medicine, 07/2010-present, Annual Direct Cost $25,000, B. Stansfield, Principal Investigator

CURRENT SUPPORT:

"Institutional startup funds", Department of Pediatrics, Augusta University, 07/01/2013-06/30/2016, Annual Direct Cost $134,000, B. Stansfield, Principal Investigator

"Characterizing Myeloid Cell Activation in NF1 Vasculopathy", Department of Defense,
07/01/2015-06/30/2018 Total Costs $606,000
B. Stansfield, Principal Investigator

"Interrogating NF1 Arterial Stenosis", American Heart Association
07/01/2015-06/30/2018 Total Costs $231,000
B. Stansfield, Principal Investigator

"Cardiovascular Abnormalities in Pediatric Patients with Neurofibromatosis Type 1", Texas Neurofibromatosis Foundation
05/01/2016 – 05/01/2017 Total Costs $44,081
Klesse, Principal Investigator, B. Stansfield, Co-Investigator

PENDING SUPPORT:

"Responsive Parenting, Sleep, and Rapid Weight Gain among African American Infants", National Institutes of Health

04/1/2017-03/31/2022 Total Costs $3,629,537
Birch, Lavner, Principal Investigator, B. Stansfield, Co-Investigator

GRANTS UNDER REVIEW:
"Mechanisms of Myeloperoxidase and NOX4 Interactions in Abdominal Aortic Aneurysm"
01/01/2018 – 12/31/2023 Total Costs $
Weintraub, Principal Investigator, B. Stansfield, Co-Investigator

PRINT AND ELECTRONIC PUBLICATIONS:

I. TEACHING AND CURRICULUM DEVELOPMENT

1. Pediatric Fellows Education Curriculum, Indiana University (2011-2013)
   Co-chaired committee to develop and implement new educational curriculum for pediatric fellows including professional development, scholarship, and quality improvement
2. PEDS 5037, Advanced Pediatrics Elective, Augusta University
   Developed curriculum and facilitated 3-day lecture series to introduce "Evidence Based Medicine" and literature searches to senior students in graduate medical education
3. VBI 8130, Modern Drug Discovery and Development, Augusta University
   Lecturer
4. Neonatal/Perinatal Medicine, Augusta University
   Developed and implemented new curriculum for graduate students, medical residents, and fellows in the Division of Neonatal/Perinatal Medicine (10 lectures/year)
5. Pediatric Resident Research Program, Chair, Augusta University
   Revised and implemented curriculum for pediatric resident research
6. Pediatric Fellow Research Program, co-leader of 1st year curriculum
   Helped develop formal didactic sessions on clinical trial design, IRB approval, protection of human subjects, statistical analysis, and manuscript preparation

II. RESEARCH, SCHOLARSHIP, OR CREATIVE ACTIVITIES (Refereed Journals)

1. Timoney PJ, **Stansfield BK**, Whitehead R, Lee HB, Nunery WR. Eyelid Lacerations Secondary to Caesarean Section Delivery. *Ophthal Plast Reconstr Surg.* 2012 Jul-Aug;28(4):e90-2.
2. **Stansfield BK**, Bessler WK, Mund JA, Downing B, Mali RS, Sarchet KN, Li F, Distasi MR, Conway SJ, Kapur R, Ingram DA. Heterozygous Inactivation of the *Nf1* Gene in Myeloid Cells Enhances Neointima Formation via a Rosuvastatin-Sensitive Cellular Pathway. *Human Molecular Genetics*. 2013 Mar 1;22(5):977-88.
3. **Stansfield BK**, Bessler WK, Mali RS, Mund JA, Downing B, Kapur R, Ingram DA. Ras-Mek-Erk Signaling Regulates *Nf1* Heterozygous Neointima Formation. *American Journal Of Pathology*. 2014 Jan;184(1):79-85.
4. Downing B, Li F, Mund JA, Bessler WB, Smiley LC, Sarchet KN, Distasi MR, Conway SJ, Clapp DW, **Stansfield BK***, Ingram DA. "Neurofibromin-deficient Myeloid Cells are Critical Mediators of Aneurysm Formation *In Vivo*". *Circulation*. 2014 Mar 129:1213-1224.
   ***Corresponding Author**
5. Ham PB, Patel P, Wise LJ, Walters C, **Stansfield BK**. "Severe Myocardial Injury and Extracorporeal Membrane Oxygenation in Perinatal Asphyxia" *J Pediatr Surg*. 2015, doi: 10.1016/j.epsc.2015.03.010

6. Ham PB, Wise LJ, Wang EJ, **Stansfield BK**, Hatley RM, Walters KC, Pipkin WL, Bhatia J. "Venovenous Extracorporeal Membrane Oxygenation for Cardiorespiratory Failure due to Congenital Diaphragmatic Hernia and Ebstein's Anomaly". *Amer. Surg.* 2015 Aug 81:9

7. **Stansfield BK***, Ingram DA. "Clinical Significance of Monocyte Heterogeneity". *Clin Transl Med*. 2015 Nov 4(1): 5 ***Corresponding Author**

8. 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.

9. **Stansfield BK***, Fain ME, Bhatia J, Gutin B, Nguyen JT, Pollock NK. "Nonlinear Relationship between Birthweight and Visceral Fat in Adolescents". *Journal of Pediatrics*. 2016 Jul;174:185-92. ***Corresponding Author**

10. Lalani A, Ham PB, Wise LJ, McDaniel JM, Walters CK, Pipkin WL, **Stansfield BK**, Hatley R, Bhatia J. "Management of Patients with Gastroschisis Requiring ECMO for Concurrent Respiratory Failure" *Amer. Surg.* 2016 Sep;82(9):768-72.

11. Christou H, Dizon MLV, Farrow K, Jadcherla SR, Leeman KT, Maheshwari A, Rubin LP, **Stansfield BK**, Rowitch DH. "Sustaining Careers of Physician-Scientists in Neonatology and Pediatric Critical Care Medicine: Formulating Supportive Departmental Policies". *Pediatric Research*. 2016 Nov;80(5):635-640.

12. Bessler WK, Hudson FZ, Zhang H, Harris V, Wang Y, Mund JA, Downing B, Ingram DA, Case J, Fulton DJ, **Stansfield BK**. "Neurofibromin Regulates Reactive Oxygen Species Production and Arterial Remodeling". *Free Radical Biology and Medicine*. 2016 Jun 3;97:212-222.

13. Masoumy, EP, **Stansfield, BK**. "Breakthrough in the Prevention of Mother-to-Child Hepatitis B Transmission?" *J Perinatology*. 2017 **37,** 333–334.

14. **Stansfield BK***, Wise LJ, Ham PB, Patel P, Parman M, Jin C, Mathur S, Harshfield G, Bhatia J. "Outcomes Following Routine Antithrombin III Replacement during Neonatal Extracorporeal Membrane Oxygenation". *Journal of Pediatric Surgery*. 2017 Apr;52(4):609-613 ***Corresponding Author**

15. Kim HW, **Stansfield BK**. "Genetic and Epigenetic Regulation of Aortic Aneurysms". *Biomed Research International.* 2017;2017(12)

16. Sawyer A, Wise LJ, Ghosh S, Bhatia J, **Stansfield BK**. "Transfusion Threshold for Neonatal ECMO". *Transfusion*. 2017 doi: 10.1111/trf.14151.

17. Yiew KH, Chatterjee TK, Tang YL, Pellenberg R, **Stansfield BK**, Bagi Z, Fulton DJ, Stepp DW, Chen W, Patel V, Kamath VM, Litwin SE, Hui DY, Rudich SM, Kim HW, Weintraub NL. "Novel role for Wnt inhibitor APCDD1 in adipocyte differentiation: implications for diet-induced obesity". *J Biol Chemist.* Apr 14;292(15):6312-6324.

18. Kim HW, Blomkalns AL, Ogbi M, Thomas M, Gavrila D, Neltner BS, Cassis LA, Thompson RW, Weiss RW, Lindower PD, Blanco VM, McCormick ML, Daugherty A,Fu X, Hazen SL, Stansfield BK, Huo Y, Chatterjee T, Weintraub NL. Role of myeloperoxidase in abdominal aortic aneurysm formation: mitigation by taurine. Submitted - Am J Physiol - Heart Circ Physiol

19. Masoumy E, Thompson JT, Zhang H, Tritz R, Richardson B, **Stansfield BK**. "Perinatal Growth Restriction Results in Irreversible Cardiomyocyte Injury and Predisposes Mice and Humans to Cardiac Dysfunction". *In Preparation.*

20. Tritz R, Haigh S, Fulton DJ, **Stansfield BK**. "Endothelial Colony Forming Cells (ECFC/EOC) Exhibit Enhanced Glycolytic Activity via the p21 Ras Pathway". *In Preparation*.

21. Benson TW, Chatterjee TK, Weintraub DS, Popoola O, Joseph J, **Stansfield BK**, Crowe M, Yiew KH, Unruh D, Pillai A, Williams J, Mintz J, Stepp DW, Brittain J, Bogdanov V, Weintraub NL. "Duffy Antigen Receptor for Chemokines Regulates Insulin Signaling and Adipocyte Maturation". *In Preparation*.

BOOK CHAPTERS:

1. **Stansfield BK**, Conway SJ, Ingram DA, Friedman JA. "Molecular Basis of Cardiovascular Abnormalities in NF1", in Upadhyay, M (ed): Neurofibromatosis Type 1. Springer-Verlag Berlin Heidelberg. 2013

ABSTRACTS PRESENTED (*PLATFORM PRESENTATION):

2000 **Stansfield BK***, Deal ST. *Regio-selective reduction of a benzylidene acetal group on a derivatized glucose,* Georgia Academy of Sciences, Valdosta, GA

2007 **Stansfield BK**, Hanevold C, Caldwell A. *Prenatal predictors of vesicoureteral reflux,* Medical College of Georgia Research Symposium, Augusta, GA

2010 **Stansfield BK**, Bessler WK, Mund JA, Ingram DA. *Nf1* heterozygous macrophages are the primary effectors of *Nf1*$^{+/-}$ neointima formation, Riley Research Symposium Indianapolis, IN

2010 **Stansfield BK***, Bessler WK, Mund JA, Sarchet KN, Downing B, Distasi M, Smiley LC, Li F, Ingram DA. *Lineage restricted studies of monocytes in Nf1*$^{+/-}$ *vaso-occlusive disease*, Midwest Section of Perinatal Medicine, Cincinnati, OH

2011 **Stansfield BK**, Bessler WK, Mund JA, Sarchet KN, Downing B, Distasi M, Li F, Ingram DA. *Nf1 heterozygous macrophages are the primary effectors of Nf1*$^{+/-}$ *neointima formation*, Riley Research Symposium, Indianapolis, IN

2011 **Stansfield BK***, Bessler WK, Mund JA, Sarchet KN, Downing B, Distasi M, Smiley LC, Li F, Ingram DA. *Heterozygous inactivation of Nf1 in monocytes/macrophages alone is necessary and sufficient for enhanced neointima formation in vivo*, Children's Tumor Foundation, Jackson Hole, WY

2012 **Stansfield BK**,* Bessler WK, Mund JA, Sarchet KN, Downing B, Distasi M, Smiley LC, Li F, Conway SJ, Kapur R, Ingram DA. *Heterozygous Inactivation of the Nf1 Gene in Myeloid Cells Enhances Neointima Formation via a Rosuvastatin-Sensitive Cellular Pathway*, Riley Research Symposium, Indianapolis, IN

2012 Downing B, Li F, Bessler WK, **Stansfield BK**, Mund JA, Sarchet KN, Distasi MC, Ingram DA. *Myeloid cells induce Nf1*$^{+/-}$ *aneurysms via activation of NADPH oxidase,* National Clinical and Translational Sciences Predoctoral Programs Meeting. Rochester, MN

2012 Downing B, Li F, Bessler WK, **Stansfield BK,** Mund JA, Distasi MC, Smiley LC, Ingram DA. *Monocyte/macrophages are the primary effectors of Nf1*$^{+/-}$ *aneurysm*

formation via increased activation of the NADPH oxidase system, Gordon Research Conference and Symposium: NOX Family NADPH Oxidases, Waterville Valley, NH

2012 **Stansfield BK***, Bessler WK, Mund JA, Downing B, Li F, Kapur R, Ingram DA. *Heterozygous Inactivation of the Nf1 Gene in Myeloid Cells Enhances Neointima Formation via a Rosuvastatin-Sensitive Cellular Pathway*, Midwest Society for Pediatric Research, Columbus, OH

2014 Bessler WK, Mali RM, Kapur R, Ingram DA, **Stansfield BK***. *MCP-1/CCR2 Signaling Mediates Nf1$^{+/-}$ Neointima Formation*, Society for Pediatric Research, Vancouver, BC

2015 **Stansfield BK***, Ingram DA. *CCR2 Signaling is Necessary for Nf1$^{+/-}$ Neointima Formation*, Southern Society for Pediatric Research, New Orleans, LA

2015 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

2015 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**

2015 **Stansfield BK**, Wise L, Patel P, Parman M, Wall D, Bhatia J. *Single Center Review of Outcomes Following Administration of Antithrombin III during Extracorporeal Membrane Oxygenation,* ELSO, Atlanta, GA

2016 **Stansfield BK**, Wise L, Patel P, Parman M, Wall D, Bhatia J. *Outcomes Following Routine Antithrombin III Replacement during Neonatal Extracorporeal Membrane Oxygenation,* Southern Society for Pediatric Research, New Orleans, LA

2016 **Stansfield BK***, Fain ME, Bhatia J, Gutin B, Nguyen JT, Pollock NK. *Nonlinear Relationship between Birthweight and Visceral Fat in Adolescents.* Southern Society for Pediatric Research, New Orleans, LA

2016 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

2016 Thompson J*, Mintz J, **Stansfield BK**. *Cardiometabolic Risk in the Offspring of Het$_{db}$ Pregnancy*, Perinatal Biology Symposium, Aspen, CO ***Perinatal Biology Travel Award**

2016 Johnston M*, Sharma N, Mathur S, **Stansfield BK**. *Addressing Childhood Obesity: One Variable at a Time*, MCG Pediatric Scholars' Day, Augusta, GA ***William P. Kanto Resident Research Award.**

2016 Sawyer A, Wise LJ, Ghosh S, Bhatia J, **Stansfield BK**. "Transfusion Threshold for Neonatal ECMO". ELSO, San Diego, CA

2016 Johnston M, Sharma N, Mathur S, **Stansfield BK**. *Addressing Childhood Obesity: One Variable at a Time*, American Academy of Pediatrics National Conference & Exhibition, San Francisco, CA

2016 Masoumy E, **Stansfield BK.** *Fetal Growth Restriction Reduces Cardiomyocyte Number and Alters Cardiac Development.* Perinatal Pediatrics, Marco Is, FL

2017 Masoumy E*, Thompson JE, Richardson BS, **Stansfield BK**. *Maternal Nutrient Restriction Programs Offspring Cardiomyocyte Fate in Guinea Pigs and Humans*. Southern Society for Pediatric Research, New Orleans, LA
*__Basic Science Young Investigator Award Finalist__

2017 Tritz R, Zhang H, Fulton DJ, **Stansfield BK**. *Metabolic Characterization of Circulating Human Endothelial Colony Forming Cells*. Southern Society for Pediatric Research, New Orleans, LA

2017 Sawyer A, Wise LJ, Bhatia J, **Stansfield BK**. *Comparison of Transfusion Thresholds for Neonatal ECMO*. Southern Society for Pediatric Research, New Orleans, LA

2017 Thompson JA, Larion S, Mintz JD, **Stansfield BK**. *Impact of Gestational Diabetes on Metabolic Risk in the Offspring*. Pediatric Academic Societies, San Francisco, CA

2017 Masoumy E*****, Thompson JE, Richardson BS, Sharma S, **Stansfield BK.** *Fetal Growth Restriction Impairs Cardiomyocyte Development in Guinea Pigs and Humans.* Pediatric Academic Societies, San Francisco, CA
***Perinatal Travel Award**

2017 Tritz R, Zhang HB, Hudson FZ, Benson TW, Kim HW, Fulton DJ, Weintraub NL, **Stansfield BK.** *Neurofibromin is a Novel Regulator of Macrophage Polarization via PFKFB3 Activation.* Children's Tumor Foundation, Washington D.C.

III. PROFESSIONAL SERVICE:

2015-present Neonatal/Perinatal Medicine Fellowship, Assistant Director

2015-2016 Southern Society for Pediatric Research, Institutional Representative

2016-present Southern Society for Pediatric Research, Council Member

IV. INTEGRATION OF TWO OR MORE ASPECTS OF FACULTY WORK:

2014-present Chair, Pediatric Resident Research Committee

Date: _____ Signature: _____