Neurofibromin Deficient Myeloid Cells are Critical Mediators of Aneurysm Formation In Vivo

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Abstract

Background—Neurofibromatosis Type 1 (NF1) is a genetic disorder resulting from mutations in the NF1 tumor suppressor gene. Neurofibromin, the protein product of NF1, functions as a negative regulator of Ras activity in circulating hematopoietic and vascular wall cells, which are critical for maintaining vessel wall homeostasis. NF1 patients have evidence of chronic inflammation resulting in development of premature cardiovascular disease, including arterial aneurysms, which may manifest as sudden death. However, the molecular pathogenesis of NF1 aneurysm formation is unknown.

Method and Results—Utilizing an angiotensin II-induced aneurysm model, we demonstrate that heterozygous inactivation of Nf1 (Nf1+/−) enhanced aneurysm formation with myeloid cell infiltration and increased oxidative stress in the vessel wall. Using lineage-restricted transgenic mice, we show loss of a single Nf1 allele in myeloid cells is sufficient to recapitulate the Nf1+/− aneurysm phenotype in vivo. Finally, oral administration of simvastatin or the antioxidant apocynin, reduced aneurysm formation in Nf1+/− mice.

Conclusion—These data provide genetic and pharmacologic evidence that Nf1+/− myeloid cells are the cellular triggers for aneurysm formation in a novel model of NF1 vasculopathy and provide a potential therapeutic target.

Keywords

- genetics
- transgenic models
- aneurysm
- leukocyte
- statin intervention
- inflammation

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Neurofibromatosis Type 1 (NF1) is an autosomal dominant genetic disorder resulting from mutations in the \textit{NF1} gene. \textit{NF1} encodes the protein neurofibromin, which negatively regulates p21\textsuperscript{Ras} (Ras) activity via stimulation of its GTPase function.\textsuperscript{1} Germline mutations causing NF1 affect only one \textit{NF1} allele, although loss of heterozygosity is described in primary tumor samples from NF1 patients.\textsuperscript{2} Thus, haploinsufficiency of \textit{NF1} results in disease with complete penetrance and diverse clinical manifestations in different organ systems.

Common non-neoplastic manifestations of NF1 include cognitive disorders and skeletal abnormalities,\textsuperscript{2} while cardiovascular disease (CVD) is a serious but under-recognized complication, contributing to significant increases in morbidity and premature mortality.\textsuperscript{3,4} In particular, the aorta and proximal branches demonstrate increased aneurysm formation and exaggerated intimal hyperplasia.\textsuperscript{5} The frequency of NF1 vasculopathy is difficult to define due to a lack of routine screening; however, the prevalence of vascular lesions in large clinical series approaches 7 percent.\textsuperscript{5–7} Specifically, a study of 31 NF1 patients with a diagnosis of vascular disease identified 38 aneurysms among the group, with an average age at diagnosis of 38 years (range: 3–77).\textsuperscript{5}

Studies utilizing mouse models that recapitulate NF1 vasocclusive disease revealed that neurofibromin-deficient myeloid cells and vascular smooth muscle cells (VSMCs) cooperate to induce neointima hyperplasia after arterial injury.\textsuperscript{8–10} Correlative studies demonstrate that NF1 patients have evidence of chronic inflammation and mobilization of a specific monocyte subset in their peripheral blood that is linked to vasocclusive disease progression and aneurysm formation in non-NF1 patients with CVD.\textsuperscript{8,11} Despite these observations, the pathogenesis of NF1 aneurysm disease is unknown, partly due to a lack of animal models that mimic the human disease. Given the mostly silent presentation of aneurysms in NF1 patients and the potential for catastrophic rupture, understanding disease pathogenesis is critical for aneurysm prevention, early detection, and treatment.

In this study, we utilized an established mouse model of aneurysm formation and cell lineage-restricted transgenic mice to test the role of \textit{Nf1} haploinsufficiency (\textit{Nf1}\textsuperscript{+/\textminus}) in various cell types on aneurysm formation. We provide evidence that heterozygous inactivation of \textit{Nf1} directly contributes to larger and more severe aneurysms with enhanced oxidative species production and matrix metalloproteinase-9 (MMP-9) activation. Further, lineage-restricted inactivation of a single \textit{Nf1} gene copy in myeloid cells, but not VSMCs, is sufficient for aneurysm formation, thereby implicating \textit{Nf1}\textsuperscript{+/\textminus} myeloid cells as the cellular triggers for \textit{Nf1}\textsuperscript{+/\textminus} aneurysm formation \textit{in vivo}. Finally, we provide pharmacologic evidence that aneurysm formation in \textit{Nf1}\textsuperscript{+/\textminus} mice is abrogated by daily low-dose administration of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor simvastatin, which has antioxidant and anti-inflammatory effects. To further delineate the pharmacologic effects of simvastatin, \textit{Nf1}\textsuperscript{+/\textminus} mice were treated with the antioxidant apocynin, which also reduced aneurysm formation. Thus, we generated a novel model of NF1-associated aneurysmal disease and provide genetic evidence that \textit{Nf1}\textsuperscript{+/\textminus} myeloid cells are critical mediators of aneurysm formation via an antioxidant-sensitive pathway, suggesting a potential therapeutic target.

\section*{Methods}

\subsection*{Animals}

All protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. \textit{Nf1}\textsuperscript{+/\textminus} mice were obtained from Tyler Jacks (Massachusetts Institute of Technology, Cambridge) and backcrossed 13 generations into...
the C57BL/6J strain. Nf1\textsuperscript{[flox/flox]} mice were obtained from Luis Parada (University of Texas Southwestern Medical Center, Dallas) and backcrossed 13 generations into the 129SvJ strain. LysM\textsuperscript{cre} (stock 4781) and SM22\textsuperscript{cre} (stock 4746) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Nf1\textsuperscript{fl/fl} mice were inter-crossed with LysM\textsuperscript{cre} or SM22\textsuperscript{cre} mice to generate F1 C57BL/6 × 129SvJ progeny. Cre-mediated recombination was confirmed by PCR.\textsuperscript{12} Lacz lineage tracing of aortas from Nf1\textsuperscript{fl/+};Rosa26\textsuperscript{fl/+};Sm22\textsuperscript{cre} mice revealed staining limited to the vessel media where VSMCs reside (data not shown). Lacz lineage tracing of aortas from Nf1\textsuperscript{fl/+};Rosa26\textsuperscript{fl/+};LysM\textsuperscript{cre} mice revealed sparse staining limited to the vessel adventitia (data not shown). Nf1\textsuperscript{fl/fl} 129SvJ mice inter-crossed with Nf1\textsuperscript{+/-} C57BL/6 mice generated F1 Nf1\textsuperscript{+/-} and WT control animals. Genotyping was performed as previously described.\textsuperscript{9}

Angiotensin II-infusion Abdominal Aortic Aneurysm (AAA) model

12 week-old control and experimental male mice were infused with Angiotensin II (AngII, 1500 ng/kg/min, Calbiochem) or saline for 35 days, as described,\textsuperscript{13} with modification. Animals were anesthetized by inhalation of 2% isoflurane, and an osmotic pump (2006, Durect Corporation) containing AngII or saline was implanted subcutaneously. At indicated time points, a portion from the aortic arch to the iliac arteries was excised for analysis.

Classification and quantification of aneurysms

Images of arteries were obtained on a stereo-microscope (Carl Zeiss Inc). The maximum external arterial diameters were measured using Metamorph 6.1 (Universal Imaging Systems Corp.). Aneurysms were defined as an increase in the external aortic diameter of >/= 50% as compared to control animals. Aortic aneurysm severity was rated from Type 0 to Type IV according to the method of Martin-McNulty et al.\textsuperscript{14} with modification: Type 0, no aneurysm; Type I, dilation 1.5 to 2 times the diameter of a normal artery; Type II, a single dilation that is more than 2 times the diameter of a normal artery; Type III, multiple dilations with the largest being 1.5 to 2 times the diameter of a normal artery; and Type IV, multiple dilations with the largest being more than 2 times the diameter of a normal artery.

Simvastatin and apocynin administration

Simvastatin (1 mg/kg/day, Besse) was administered in water via oral gavage beginning 7 days prior to AngII or saline infusion and continued throughout the experiment. Apocynin (also known as acetovanillone, 100 mg/kg/day, Acros Organics) was administered in drinking water beginning 7 days prior to AngII or saline infusion and continued throughout the experiment. Control mice received water at a similar volume.

Statistical analysis

Quantitative results are shown as mean ± SEM. All statistical analyses were performed using Prism 5 (GraphPad Software). P values were obtained by the unpaired-Student’s \textit{t}-test when comparing 2 groups and by one-way analysis of Variance (ANOVA) followed by Tukey’s analysis when comparing 3 or more groups. P<0.05 were considered significant. To determine significance of categorical data, Fisher’s exact test was used with Bonferonni correction.

Results

Heterozygous inactivation of \textit{Nf1} amplifies the incidence and severity of aneurysm formation in angiotensin II-infused mice

To test the hypothesis that \textit{Nf1} heterozygosity enhances aneurysm formation \textit{in vivo}, wild-type (WT) and \textit{Nf1}\textsuperscript{+/-} mice were infused with AngII to induce aneurysms. Infusion of AngII
induces inflammatory mediators and reactive oxygen species (ROS) within the arterial wall, producing abdominal aortic aneurysms (AAA) that recapitulate human lesions.\(^{15}\)

Infusion of AngII increased aneurysm formation 3-fold in Nf1\(^{+/−}\) mice compared to WT mice (Figure 1A and 1B). Morphometric analysis of abdominal aortas from both genotypes revealed that AngII-infused Nf1\(^{+/−}\) mice had significantly larger aneurysms (Figure 1C and 2A). Further, Nf1\(^{+/−}\) aneurysms were more severe than WT aneurysms quantitatively, with increased degradation of the elastic lamina and disorganized architecture, which is reminiscent of lesions from NFI patients (Figure 1D, 2B and 2D).\(^{16}\) Importantly, AngII infusion did not alter body weight or intra-arterial blood pressure in either genotype (data not shown). Saline-infused Nf1\(^{+/−}\) and WT mice did not form aneurysms (Figure 1A, 1B and 2A). These data indicate Nf1 heterozygosity augments AngII-induced aneurysm formation.

Nf1\(^{+/−}\) aneurysms are characterized by inflammatory cell infiltration, VSMC expansion, and ROS production

NF1 patients and Nf1\(^{+/−}\) mice have increased populations of circulating inflammatory monocytes and pro-inflammatory cytokines linked to aneurysm formation.\(^{8,9}\) Therefore, we sought to characterize the cellular and structural composition of WT and Nf1\(^{+/−}\) aneurysms. Histologic examination of Nf1\(^{+/−}\) aneurysms demonstrated significant dilation and degradation of the aorta, including increased disruption of the elastic lamina and adventitial expansion when compared to WT aneurysms (Figure 2A through 2E). Importantly, Nf1\(^{+/−}\) aneurysms contained 4-times the number of macrophages compared to WT (Figure 2C and 2E). Infiltrating T cells, mast cells, and neutrophils were increased in both Nf1\(^{+/−}\) and WT aneurysms, but accounted for less than 5% of all cells and did not differ between genotypes (data not shown). Importantly, Nf1\(^{+/−}\) macrophages co-localized to sites of elastic lamina degradation, medial rupture and adventitial expansion, indicating their potential role in aneurysm formation (Figure 2B and 2C). AngII infusion also induced a significant expansion of VSMCs in the media of Nf1\(^{+/−}\) aortas compared to WT (Supplemental Figure 1A and 1B). Nf1\(^{+/−}\) VMSC expansion is consistent with previous findings that Nf1\(^{+/−}\) VSMCs exhibit increased proliferation and migration in response to cytokines secreted by macrophages and vascular wall cells implicated in CVD.\(^{9,17}\) Finally, co-staining with α-smooth muscle actin and anti-Mac-3 illustrated VSMC expansion was within and near the vessel media while macrophage infiltration was primarily in the adventia (Supplemental Figure 1C).

Genetic studies demonstrate that VSMC and macrophage secretion of MMPs and ROS are important molecular triggers for extracellular matrix (ECM) remodeling and aneurysm induction.\(^{18}\) Given the increased density of VSMCs and macrophages in Nf1\(^{+/−}\) aneurysms, we measured expression and activation of MMP-2 and MMP-9 in aortas harvested from Nf1\(^{+/−}\) and WT mice infused with AngII or saline. AngII infusion increased both MMP-2 and MMP-9 activity and preferentially amplified MMP-9 expression in Nf1\(^{+/−}\) aneurysms when compared with AngII-infused WT aneurysms as determined by \textit{in situ} zymography and immunohistochemistry (IHC) (Figure 3A). In addition, gelatin zymography of abdominal aortic explants from AngII-infused WT and Nf1\(^{+/−}\) mice corroborated increases of MMP-2 and MMP-9 in both genotypes with significantly enhanced activation of MMP-9 in Nf1\(^{+/−}\) aortas (Figure 3B). Amplified MMP-9 expression in Nf1\(^{+/−}\) aortas is an important observation since MMP-9 is largely derived from vessel wall macrophages,\(^{19}\) which is consistent with the increased macrophage infiltration observed in Nf1\(^{+/−}\) aneurysms.

We next assessed ROS production in abdominal aortic cross-sections from AngII and saline-infused Nf1\(^{+/−}\) and WT mice with dihydroethidium (DHE), a superoxide probe. AngII infusion significantly increased superoxide production in Nf1\(^{+/−}\) aortas when compared with
WT aortas, while superoxide production was nearly undetectable in the aortas from saline-infused \( Nf1^{+/−} \) and WT aortas (Figure 3C and 3D). Collectively, these data demonstrate that \( Nf1 \) heterozygous aneurysms have evidence of increased inflammatory cell infiltration, MMP activation, and ROS production, which are linked to abnormal arterial remodeling and disease progression.

**Heterozygous inactivation of \( Nf1 \) in myeloid cells alone is sufficient to recapitulate \( Nf1^{+/−} \) aneurysm formation**

\( Nf1^{+/−} \) mice have increased aneurysm formation characterized by increased macrophages and VSMCs and their secretory products that promote disease progression. Based on these observations, we generated transgenic mice with a single copy of the \( Nf1 \) gene ablated in VSMCs or myeloid cells alone to determine the role of \( Nf1 \) heterozygosity in VSMCs and macrophages on aneurysm formation. Briefly, \( Nf1^{0/0} \) mice containing conditional \( Nf1 \) alleles susceptible to Cre-mediated recombination were inter-crossed with \( SM22cre \) or \( LysMcre \) transgenic mice, generating \( Nf1^{0/+};SM22cre \) and \( Nf1^{0/+};LysMcre \) progeny. \( Nf1^{0/0} \) mice underwent efficient Cre-mediated recombination when crossed with \( LysMcre \) (Supplemental Figure 2F) or \( SM22cre \) mice. \(^{10} \) \( LysMcre \)-mediated recombination was seen in the aortic adventia, a known location for macrophages, while the aortic media showed minimal recombination.

\( Nf1^{0/0};SM22cre \) and \( Nf1^{0/+};LysMcre \) mice were infused with AngII or saline and evaluated for aneurysm formation along with littermate controls (WT and \( Nf1^{+/−} \) mice). \( Nf1^{0/0};LysMcre \) mice infused with AngII developed large aneurysms recapitulating the phenotype of \( Nf1^{+/−} \) mice, while \( Nf1^{0/0};SM22cre \) mice produced less severe aneurysms similar to WT mice (Figure 4A through 4D). Specifically, a 2.5-fold increase in AAA incidence was observed in \( Nf1^{0/0};LysMcre \) mice compared with \( Nf1^{0/0};SM22cre \) and WT mice (Figure 4B). Saline infusion failed to produce aortic aneurysms in all genotypes (data not shown). Additionally, AngII-infused \( Nf1^{0/0};LysMcre \) aneurysms displayed similar maximal dilation and aneurysm severity when compared to \( Nf1^{+/−} \) mice (Figure 4C and 4D).

Histologic examination of H&E and van Gieson stained arterial cross-sections of \( Nf1^{0/0};LysMcre \) aneurysms revealed increased elastic lamina degradation and adventitial expansion, similar to aneurysms harvested from AngII-infused \( Nf1^{+/−} \) mice (Supplemental Figure 2A through 2C). Cross-sections from \( Nf1^{0/0};LysMcre \) aortas demonstrated increased macrophage density similar to \( Nf1^{+/−} \) aneurysms, while \( Nf1^{0/0};SM22cre \) and WT mice contained significantly reduced macrophage numbers (Figure 4E). Similar to \( Nf1^{+/−} \) mice, macrophages in \( Nf1^{0/0};LysMcre \) mice were in close proximity to areas of adventitial expansion and elastic lamina degradation (Figure 4E and Supplemental Figure 2B). Additionally, \( Nf1^{0/0};LysMcre \) and \( Nf1^{0/0};SM22cre \) displayed similar MMP activity and DHE staining as \( Nf1^{+/−} \) and WT mice, respectively (Supplemental Figure 2D and 2E). Collectively, these data provide genetic evidence that heterozygous inactivation of \( Nf1 \) in myeloid cells alone is sufficient to recapitulate \( Nf1^{+/−} \) aneurysm formation in vivo, thereby implicating \( Nf1^{+/−} \) macrophages as the cellular trigger for aneurysm formation.

**Simvastatin attenuates AngII-induced AAA formation in \( Nf1^{+/−} \) mice**

HMG-CoA reductase inhibitors are clinically efficacious in the prevention of several manifestations of CVD, including aneurysm formation,\(^{20} \) which is in part attributable to their anti-inflammatory and antioxidant function.\(^{21} \) Recent studies have shown that daily statin therapy reduces arterial stenosis in \( Nf1^{+/−} \) mice, in part by inhibiting macrophage functions central to disease progression.\(^{9} \) Therefore, we tested whether simvastatin would reduce \( Nf1^{+/−} \) aneurysm formation given our experimental observations.
WT and Nf1+/− mice were treated with daily low-dose simvastatin (1 mg/kg/day) or water for 7 days prior to initiation of AngII infusion and continued for 35 days. Low-dose simvastatin reduced AAAs in AngII-infused Nf1+/− mice by greater than 2-fold compared to water-treated controls (Figure 5A and 5B) without affecting blood pressure or serum cholesterol levels (data not shown). Corresponding decreases in aortic diameter and severity in AngII-infused Nf1+/− mice were also observed (Figure 5C and 5D). There was no significant difference in AAA incidence, maximum aortic diameter, or severity in AngII-infused WT mice in either treatment group (Figure 5A through 5D). Further, simvastatin treatment reduced arterial remodeling, macrophage infiltration (Supplemental Figure 3A and 3B), MMP-9 expression and activation, and ROS production (Supplemental Figure 4A through 4D) in arterial cross-sections from Nf1+/− mice when compared to water treatment. These results demonstrate that simvastatin prevents AngII-induced AAA formation in Nf1+/− mice, providing a potential therapeutic for NF1 aneurysmal disease.

Apocynin attenuates AngII-induced AAA formation in Nf1+/− mice

Increased production of ROS has been demonstrated in several animal models of CVD and antioxidant therapy has shown some utility in reversing many of these processes. Based on our observation that Nf1+/− aortas have evidence of increased ROS in response to AngII infusion, we sought to explore the role of antioxidant therapy, using apocynin, in attenuating Nf1+/− aneurysm formation. Though generally recognized as a non-specific antioxidant, recent evidence suggests that apocynin may inhibit superoxide production in cells containing myeloperoxidase, including macrophages and monocytes.

WT and Nf1+/− mice were treated with apocynin for 7 days prior to initiation of AngII infusion and continued for 35 days thereafter. Apocynin reduced AAAs in AngII-infused Nf1+/− mice by greater than 2-fold compared to water-treated controls, while apocynin did not have a significant effect on WT mice (Figure 6A and 6B). Additionally, decreases in both maximum abdominal aortic diameter and aneurysm severity were also noted in apocynin-treated Nf1+/− mice while apocynin-treated WT mice did not display a difference in either parameter (Figure 6C and 6D). Remodeling of the arterial wall, macrophage infiltration and ROS production was significantly reduced in apocynin-treated Nf1+/− mice when compared to water-treated controls (Supplemental Figure 5A–5D). These results identify overproduction of ROS as a significant contributor to Nf1+/− aneurysm formation and provide evidence that antioxidants may be a viable therapeutic option.

Discussion

Cardiovascular disease is a non-neoplastic manifestation in NF1 patients, which contributes to debilitating morbidities and early mortality. Many of these vascular pathologies, including aneurysm formation and arterial stenosis, are often clinically silent until a catastrophic event, making an accurate measure of disease burden difficult to determine. Thus, understanding the pathogenesis of NF1 vasculopathy is critical to facilitate appropriate CVD screening, early recognition and targeted intervention in NF1 patients.

A major limitation in understanding NF1 aneurysmal disease has been the lack of an animal model that closely recapitulates the human disease. In this study, we present a murine model of NF1 aneurysmal disease, which provides a novel approach to examine the cellular mechanisms that regulate NF1 aneurysm formation. Analysis of Nf1+/− aneurysms revealed increased macrophage infiltration, MMP-9 expression and activation, and ROS production, which are molecular and cellular signatures of aneurysm formation observed in other experimental animal models independent of neurofibromin deficiency. These observations suggest that vascular inflammation and macrophage secretory products are critical factors in NF1 aneurysmal disease, which is consistent with aneurysm formation in
other chronic inflammatory diseases.\textsuperscript{25, 26} This is an important observation since we previously demonstrated that NF1 patients without known CVD have increased numbers of a specific subset of circulating inflammatory monocytes and cytokines,\textsuperscript{8} which have been previously linked to vascular disease in non-NF1 subjects in large population studies.\textsuperscript{11}

To determine the contribution of neurofibromin-deficient myeloid cells to NF1\textsuperscript{+/-} aneurysm formation, we utilized lineage-restricted transgenic mice to specifically ablate a single Nf1 allele in myeloid cells alone. Heterozygous inactivation of the Nf1 gene in myeloid cells was sufficient to reproduce the aneurysm phenotype observed in NF1\textsuperscript{+/-} mice infused with AngII. Importantly, despite significant expansion of NF1\textsuperscript{+/-} VSMCs in arterial walls, aneurysm frequency and severity in transgenic mice harboring a single Nf1 allele in VSMCs alone were similar to WT controls. Additionally, administration of simvastatin, a statin with potent anti-inflammatory and antioxidant effects, diminished aneurysm formation in NF1\textsuperscript{+/-} mice. Finally, the antioxidant apocynin efficiently reduced NF1\textsuperscript{+/-} aneurysm formation. Collectively, these data directly implicate neurofibromin-deficient myeloid cells as the critical cellular effectors of aneurysm formation in NF1\textsuperscript{+/-} mice and indicate ROS as a therapeutic target to prevent or treat NF1 aneurysm formation.

Neurofibromin negatively regulates the Ras signaling cascade in multiple cell types by accelerating the conversion of active Ras-GTP to its inactive GDP confirmation.\textsuperscript{1} Loss of neurofibromin activates Ras and its downstream effectors, including the Ras-Mek-Erk and Ras-PI-3K pathways, rendering cells hypersensitive to diverse growth factors, contributing to the complexity of disease manifestations observed in NF1 patients.\textsuperscript{2, 8, 9, 17} Interestingly, myeloid progenitor cells are particularly sensitive to Ras activation and demonstrate multiple gain-of-function phenotypes contributing to myelo-proliferative disease, plexiform neurofibroma formation, bone disease, and vasocclusive disease in animal models of NF1 disease.\textsuperscript{8, 9, 27–29} Relevant to the current study, myeloid cells secrete growth factors and cytokines that are mediators of CVD, including vessel occlusion and aneurysm formation.\textsuperscript{9, 30} In view of our recent report that neurofibromin-deficient myeloid cells are the primary mediators of NF1\textsuperscript{+/-} arterial stenosis,\textsuperscript{9} the observation that mice with heterozygous inactivation of Nf1 in myeloid cells alone form aneurysms at a similar incidence to NF1\textsuperscript{+/-} mice highlights the global pathogenic consequences of neurofibromin-deficient myeloid cells to diverse NF1 clinical manifestations, including CVD.

Myeloid cell recruitment and infiltration of the vessel wall to initiate elastic lamina degradation are essential steps in aneurysm formation.\textsuperscript{25} AngII facilitates aneurysm formation via activation of monocytes and other leukocytes, which secrete cytokines and chemotactic factors leading to enhanced macrophage production of MMPs, resulting in vascular inflammation and vessel wall remodeling.\textsuperscript{31} Though several molecular ligand-receptor signaling cascades contribute to the progression of aneurysmal disease, pharmacologic inhibition or genetic disruption of monocyte chemotactic protein-1 (MCP-1) binding to its primary receptor, CCR2, reduces aneurysm formation as well as MMP-2 and MMP-9 activation.\textsuperscript{31–33} This signaling axis is particularly interesting since our laboratory recently demonstrated that myeloid cell heterozygosity mobilized Ly6Ch\textsuperscript{hi} monocytes in peripheral blood,\textsuperscript{9} which are the murine correlate of human pro-inflammatory monocytes and co-express high cell surface levels of the CCR2 receptor.\textsuperscript{34, 35} Whether NF1\textsuperscript{+/-} macrophages are mobilized from the bone marrow via the MCP-1/CCR2 axis or proliferate locally within the aortic wall from the recently described common myeloid progenitor remains to be elucidated.\textsuperscript{31, 36}

Another striking observation in our study is the increased production of ROS and MMPs in NF1\textsuperscript{+/-} vessel walls and developing aneurysms. ROS and MMP production by various cell types is critical for aneurysm formation in several model systems. Genetic or pharmacologic
disruption of MMP-2 and MMP-9 in mice decreases aneurysm formation. These findings suggest that MMPs, released by resident and infiltrating vascular wall cells, are key molecular events in aneurysm formation. Given these observations, our study suggests that increased levels of MMP-9 observed in Nf1 heterozygous aneurysms may play a significant role in NF1 aneurysm progression, warranting further investigation to examine whether genetic modification and pharmacologic inhibition of MMP-9 activity can inhibit aneurysm progression in our Nf1 experimental system.

Increased ROS levels are also detected in human and murine cardiovascular lesions including aneurysms. Moreover, evidence suggests that ROS overproduction facilitates MMP activation and contributes to vascular inflammation and vessel wall remodeling. Neurofibromin directly stimulates the adenylyl cyclase/cyclic AMP pathway, while loss of neurofibromin amplifies mitochondrial ROS production in Drosophila melanogaster. Conversely, constitutively active Ras mutations dramatically increase ROS production in mammalian hematopoietic progenitor cells via NAD(P)H oxidase (NOX) activation without increasing mitochondrial ROS. Additionally, we have found that simvastatin and apocynin, which both have antioxidant properties, reduce Nf1 aneurysms, implicating oxidative stress as a major contributor to neurofibromin-deficient aneurysm formation. Further investigation using complex transgenic mice and targeted pharmacotherapies will now be needed to explore the redox balance in neurofibromin-deficient cells and to determine the source of ROS in aneurysm pathogenesis.

Previous studies have also identified increased expression of ROS-producing NOX subunits p22phox and p47phox within aortic aneurysms of non-NF1 patients and mice. Genetic disruption of p47phox significantly diminishes oxidative stress and subsequent aneurysm formation in mice infused with AngII. These data suggest a role of NOX proteins as producers of ROS in aneurysm pathogenesis, indicating potential sources for observed Nf1 ROS production. Interestingly, Heumuller et al. extensively studied apocynin’s ability to reduce ROS in infiltrating and resident vascular wall cells, concluding that apocynin is dimerized and activated in myeloperoxidase expressing cells, including macrophages and granulocytes. Apocynin’s dimerized form is believed to inhibit the binding of the p47phox cytosolic subunit to the membrane-bound gp91phox subunit, therefore inhibiting the formation of the active NOX2 complex. These studies provide rationale for transgenic murine studies using cell specific deletion of p47phox and gp91phox within endothelial cells, VSMCs and monocytes/macrophages in Nf1 mice to test whether specific NOX isoform(s) contribute to ROS overproduction and aneurysm development. These studies are currently underway in our laboratory to identify specific therapeutic targets for preventing and treating NF1 vascular disease.

Statins are efficacious in reducing inflammation and oxidative stress independent of their lipid lowering capacity in both human trials and animal models. Additionally, statins have a safety profile that makes them advantageous for use in pediatric patients, as evidenced by recent trials in NF1 children, which may be important since evidence of vascular inflammation was identified in adolescent NF1 patients. In the current study, simvastatin reduced Nf1 aneurysm formation with corresponding attenuation of MMP-9 activation and ROS production, lending evidence to the role of inflammation and oxidative stress in NF1 aneurysm development. Finally, treatment with the antioxidant apocynin, which produced similar results to simvastatin, indicates that a reduction of oxidative stress may mediate the therapeutic effect simvastatin has on reducing Nf1 aneurysm formation. Based on our pre-clinical findings that low-dose statin treatment attenuates aneurysm formation and vasocclusive disease, it is possible that statins or more general antioxidants could be a viable therapeutic intervention in NF1 patients for the prevention and treatment of CVD.
In sum, this study establishes the first animal model of NF1 aneurysm disease and identifies \textit{Nf1} heterozygous myeloid cells as the cellular effectors of \textit{Nf1} \textit{+}/\textit{−} aneurysm formation. In addition, we provide significant evidence that oxidative stress partially mediates \textit{Nf1} \textit{+}/\textit{−} aneurysm formation and may be a viable therapeutic target. We provide a new model of NF1 vasculopathy that will serve as a tractable platform for understanding disease pathogenesis, the identification of novel biomarkers of pre-clinical disease, and development of novel therapeutics for the prevention and/or treatment of NF1 aneurysm formation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Figure 1. 
*Nf1*+/− mice have enhanced AngII-induced AAA formation. (A) Representative photographs of the aorta and branches from saline or AngII-infused WT and *Nf1*+/− mice. Boxes identify area magnified in lower panel. Scale bars: 1mm. (B) Quantification of aneurysm incidence. *P*<0.0083 for saline-infused WT (n=24) versus AngII-infused WT (n=29) and AngII-infused *Nf1*+/− (n=31). **P*<0.0083 for saline-infused *Nf1*+/− (n=16) versus AngII-infused *Nf1*+/−. Analysis by Fisher’s exact test with Bonferroni correction. (C) Maximum abdominal aortic diameter of saline or AngII-infused WT and *Nf1*+/− mice. Clustering around 1mm represents animals without aneurysm formation. *P*<0.05 for saline-infused *Nf1*+/− (n=16) versus AngII-infused *Nf1*+/− (n=31). **P*<0.05 for AngII-infused WT (n=29) versus AngII-infused *Nf1*+/− (n=31). No statistical significance was observed for saline-infused WT (n=24) versus AngII-infused WT (n=29). Analysis by one-way ANOVA with Tukey’s test. Error bars denote the mean ± SEM. (D) Aneurysm severity for AngII-infused WT (n=29) or AngII-infused *Nf1*+/− (n=31) mice. No aneurysms formed in saline-infused mice of either genotype.
Figure 2.
Histological and morphometric analysis of abdominal aortas from WT and Nf1<sup>+/−</sup> mice. (A) Representative photomicrographs of abdominal aortic cross-sections from saline and AngII-infused WT and Nf1<sup>+/−</sup> mice stained with H&E, (B) van Gieson or (C) anti-Mac-3 antibody (arrowheads). Boxed areas magnified in lower panel. Arrowheads in B indicate elastic lamina fragmentation. Scale bars: 50 μm. (D) Grading of elastic lamina degradation in saline or AngII-infused WT and Nf1<sup>+/−</sup> mice. *P<0.05 for saline-infused Nf1<sup>+/−</sup> (n=5) or WT (n=5) versus AngII-infused Nf1<sup>+/−</sup> mice (n=13). **P<0.05 for AngII-infused WT (n=15) versus AngII-infused Nf1<sup>+/−</sup> mice. No statistical significance was observed for saline-
infused WT or Nf1+/− versus AngII-infused WT. (E) Quantification of Mac3-positive macrophages per high-power field (HPF) in saline or AngII-infused WT and Nf1+/− mice. *P < 0.05 for saline-infused Nf1+/− (n=5) versus AngII-infused Nf1+/− mice (n=5).
**P<0.05 for AngII-infused WT (n=5) versus AngII-infused Nf1+/− mice. No statistical significance was observed for saline-infused WT (n=5) versus AngII-infused WT. Analysis by one-way ANOVA with Tukey’s test.
Figure 3.
AngII induces MMP-9 expression and activity and ROS production in Nf1+/− mice. (A) Representative photomicrographs of abdominal aortic cross-sections from saline and AngII-infused WT and Nf1+/− mice. MMP activity (green) was visualized by in situ zymography and expression of MMP-2 and MMP-9 was detected by IHC staining with anti-MMP-2 (brown) and anti-MMP-9 (brown) antibodies. Scale bars: 50 μm. (B) Representative zymogram showing abdominal aortic MMP-2 and MMP-9 levels for saline and AngII-infused WT and Nf1+/− mice. (C) Representative photomicrographs of abdominal aortic cross-sections from saline or AngII-infused WT and Nf1+/− mice, showing superoxide production identified by in situ DHE staining (red). Auto-fluorescence of murine tissue is visible (green). (D) Quantification of ethidium fluorescence. *P<0.05 for AngII-infused
$Nf1^{+/−}$ ($n=9$) versus saline-infused WT ($n=5$) and $Nf1^{+/−}$ ($n=3$). **$P<0.05$ for AngII-infused $Nf1^{+/−}$ versus AngII-infused WT ($n=8$).
Figure 4.
Heterozygous inactivation of Nf1 in myeloid cells alone is sufficient to recapitulate Nf1 aneurysm formation. (A) Representative photographs of abdominal aortas from AngII-infused WT, Nf1+/−, Nf1floxed−/−;LysMcre and Nf1floxed−/−;SM22cre mice. Scale bars: 1mm. Saline-infused WT, Nf1+/−, Nf1floxed−/−;LysMcre and Nf1floxed−/−;SM22cre mice did not form aneurysms (data not shown). (B) Quantification of aneurysm incidence. (C) Maximum abdominal aortic diameter of AngII-infused WT, Nf1+/−, Nf1floxed−/−;LysMcre and Nf1floxed−/−;SM22cre mice. Clustering around 1mm represents animals without aneurysm formation. *P<0.05 for AngII-infused WT (n=10) versus AngII-infused Nf1+/− (n=9), and
AngII-infused WT versus $Nf1^{flox/+};LysMcre$ ($n=15$). **$P<0.05$ for AngII-infused $Nf1^{flox/+};SM22cre$ ($n=10$) versus AngII-infused $Nf1^{+/-}$, and AngII-infused $Nf1^{flox/+};SM22cre$ versus AngII-infused $Nf1^{flox/+};LysMcre$. Analysis by one-way ANOVA with Tukey’s test. Error bars denote mean ± S.E.M. For B and C, no statistical significance was observed for AngII-infused WT versus AngII-infused $Nf1^{flox/+};SM22cre$, or AngII-infused $Nf1^{+/-}$ versus AngII-infused $Nf1^{flox/+};LysMcre$. (D) Severity index of aneurysms for AngII-infused WT ($n=10$), $Nf1^{+/-}$ ($n=9$), $Nf1^{flox/+};LysMcre$ ($n=15$) and $Nf1^{flox/+};SM22cre$ mice ($n=10$). (E) Representative photomicrographs of abdominal aortic cross-sections from AngII-infused WT, $Nf1^{+/-}$, $Nf1^{flox/+};LysMcre$, and $Nf1^{flox/+};SM22cre$ mice stained with anti-Mac-3 (arrowheads). Boxes specify area magnified in lower panel. Scale bars: 50μm.
Figure 5.
Preventive effect of simvastatin on AngII-induced AAA formation in Nf1+/− mice. (A) Representative photographs of abdominal aortas from water or simvastatin-treated, AngII-infused WT and Nf1+/− mice. Scale bars: 1mm. (B) Quantification of aneurysm incidence in water or simvastatin-treated, AngII-infused mice. (C) Maximum abdominal aortic diameter of water or simvastatin-treated, AngII-infused WT and Nf1+/− mice. Clustering around 1mm represents animals without aneurysm formation. *P<0.05 for water-treated WT (n=17) versus water-treated Nf1+/− (n=22). **P<0.05 for water-treated Nf1+/− versus simvastatin-treated Nf1+/− (n=10). Analysis by one-way ANOVA with Tukey’s test. Error bars denote
the mean ± S.E.M. For B and C, no statistical significance was observed for water-treated WT versus simvastatin-treated WT (n=13). (D) Severity index of AAAs of AngII-infused WT and Nf1+/− mice treated with water (WT, n=17; Nf1+/−, n=22) or simvastatin (WT, n=9; Nf1+/−, n=10). For B–D, saline-infused WT or Nf1+/− mice in either treatment group did not form aneurysms.
Figure 6.
Preventive effect of apocynin on AngII-induced AAA formation in Nf1+/− mice. (A) Representative photographs of abdominal aortas from water or apocynin-treated, AngII-infused WT and Nf1+/− mice. Scale bars: 1mm. (B) Quantification of aneurysm incidence in water or apocynin-treated, AngII-infused mice. *P<0.0083 for water-treated Nf1+/− (n=23) versus apocynin-treated Nf1+/− (n=26). Analysis by Fisher’s exact test with Bonferroni Correction. (C) Maximum abdominal aortic diameter of water or apocynin-treated, AngII-infused WT and Nf1+/− mice. Clustering around 1mm represents animals without aneurysm formation. *P<0.05 for water-treated WT (n=14) versus water-treated Nf1+/− (n=23).
**P<0.05 for water-treated Nf1+/− versus apocynin-treated Nf1+/− (n=26). Analysis by one-way ANOVA with Tukey’s test. Error bars denote the mean ± S.E.M. For B and C, no statistical significance was observed for water-treated WT versus apocynin-treated WT (n=24). (D)Severity index of AAAs of AngII-infused WT and Nf1+/− mice treated with water (WT, n=14; Nf1+/−, n=23) or apocynin (WT, n=24; Nf1+/−, n=26). For B-D, saline-infused WT or Nf1+/− mice in either treatment group did not form aneurysms.