Heterozygous inactivation of the *Nf1* gene in myeloid cells enhances neointima formation via a rosuvastatin-sensitive cellular pathway

Brian K. Stansfield^{1,2}, Waylan K. Bessler^{1,2}, Raghuveer Mali^{1,2}, Julie A. Mund^{1,2}, Brandon Downing^{1,2}, Fang Li^{1,2}, Kara N. Sarchet^{1,2}, Matthew R. DiStasi^{1,2}, Simon J. Conway^{1,2,3}, Reuben Kapur^{1,2,3} and David A. Ingram Jr^{1,2,3,*}

¹Herman B. Wells Center for Pediatric Research, ²Department of Pediatrics and Neonatal-Perinatal Medicine, and ³Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, IN 46202, USA

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Mutations in the *NF1* tumor suppressor gene cause Neurofibromatosis type 1 (NF1). Neurofibromin, the protein product of *NF1*, functions as a negative regulator of Ras activity. Some NF1 patients develop cardiovascular disease, which represents an underrecognized disease complication and contributes to excess morbidity and mortality. Specifically, NF1 patients develop arterial occlusion resulting in tissue ischemia and sudden death. Murine studies demonstrate that heterozygous inactivation of *Nf1* (*Nf1*^{+/-}) in bone marrow cells enhances neointima formation following arterial injury. Macrophages infiltrate *Nf1*^{+/-} neointimas, and NF1 patients have increased circulating inflammatory monocytes in their peripheral blood. Therefore, we tested the hypothesis that heterozygous inactivation of *Nf1* in myeloid cells is sufficient for neointima formation. Specific ablation of a single copy of the *Nf1* gene in myeloid cells alone mobilizes a discrete pro-inflammatory murine monocyte population via a cell autonomous and gene-dosage dependent mechanism. Furthermore, lineage-restricted heterozygous inactivation of *Nf1* in myeloid cells is sufficient to reproduce the enhanced neointima formation observed in *Nf1*^{+/-} mice when compared with wild-type controls, and homozygous inactivation of *Nf1* in myeloid cells amplified the degree of arterial stenosis after arterial injury. Treatment of *Nf1*^{+/-} mice with rosuvastatin, a statin with anti-inflammatory properties, significantly reduced neointima formation when compared with control. These studies identify neurofibromin-deficient myeloid cells as critical cellular effectors of *Nf1*^{+/-} neointima formation and propose a potential therapeutic for NF1 cardiovascular disease.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease affecting 1 in 3500 individuals. NF1 results from mutations in the *NF1* tumor suppressor gene that encodes the protein neurofibromin (1). Neurofibromin is a GTPase-activating protein for Ras and negatively regulates Ras signaling (2,3). Whereas loss of *NF1* heterozygosity is described in primary tumor samples, germline mutations that cause NF1 affect only one copy of the *NF1* gene. Haploinsufficiency of *NF1* results in disease with complete penetrance and a range of neoplastic and non-neoplastic manifestations.

Common non-neoplastic manifestations of NF1 include skeletal abnormalities, hearing loss and cognitive deficits. One of the least studied manifestations of NF1 involves disorders of the cardiovascular system, which represent an underrecognized disease complication and contribute to excess morbidity and mortality, particularly among younger patients (4–9). Specifically, NF1 patients develop intimal hyperplasia, characterized by vascular smooth muscle cell (VSMC) accumulation and vessel lumen stenosis resulting in cerebral and visceral ischemia and infarction (4–9).

Neurofibromin is a regulator of endothelial cell (EC), VSMC and hematopoietic cell function in response to multiple

*To whom correspondence should be addressed at: Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, 1044 W. Walnut St R4/470, IN 46202, USA. Tel: +317 2788245; Fax: +317 2748679; Email: dingram@iupui.edu; janiwall@iupui.edu

growth factors implicated in neointima formation (10–13). However, the lineage-specific regulatory function of neurofibromin in the coordination of ECs, VSMCs and circulating hematopoietic cells to maintain blood vessel wall homeostasis *in vivo* remains poorly understood. Previous data demonstrate that *Nf1*^{+/-} mice form enlarged neointimas characterized by proliferating VSMCs, infiltration of inflammatory macrophages and amplification of Ras-ERK signaling in response to arterial injury (14).

Utilizing *cre/lox* technology, we previously demonstrated that cell-specific loss of a single *Nf1* allele in ECs and/or VSMCs failed to induce neointima formation after arterial injury (15). However, lethally irradiated wild-type (WT) mice reconstituted with *Nf1*^{+/-} hematopoietic stem cells uniformly developed enlarged neointimas after arterial injury similar to *Nf1*^{+/-} mice with germline heterozygous inactivation of *Nf1* (15). These data provide genetic and cellular evidence that bone marrow-derived *Nf1*^{+/-} hematopoietic cells are sufficient for neointima formation after vessel injury. However, the hematopoietic cell lineage responsible for this NF1 vascular phenotype remains unknown. Identification of this cell population is important for understanding disease pathogenesis and development of experimental therapeutics and potential biomarkers of disease progression.

Although multiple hematopoietic cell lineages contribute to neointima formation, several lines of experimental evidence suggest that *Nf1*^{+/-} myeloid cells are potentially important for neointima formation *in vivo*. *Nf1*^{+/-} macrophages are abundant in *Nf1*^{+/-} murine neointimas and neurofibromin is a critical regulator of Ras-ERK signaling in multiple myeloid cell subtypes, including myeloid progenitors, osteoclasts and mature differentiated macrophages (15,16). Furthermore, in human corollary studies, analysis of peripheral blood from NF1 patients without overt signs of cardiovascular disease shows a significant increase in the number of circulating CD14^{dim}CD16^{hi} monocytes and the levels of pro-inflammatory cytokines IL-1 β , IL-6 and fractalkine, which have been previously linked to cardiovascular disease in other patient populations (15).

Based on these observations, we employed *cre/lox* technology to generate mice that were either heterozygous or homozygous for *Nf1* in myeloid cells alone to directly test the hypothesis that neurofibromin-deficient myeloid cells are the critical hematopoietic cell effectors of neointima formation after vessel injury *in vivo*. We further tested whether rosuvastatin, a statin with anti-inflammatory properties (17–19), could prevent or diminish neointima formation in *Nf1*^{+/-} mice.

RESULTS

Genetic ablation of *Nf1* in myeloid cells increases circulating Ly6C^{hi} monocytes via a cell autonomous and gene dosage-dependent mechanism

NF1 patients demonstrate increased mobilization of CD14^{dim}CD16^{hi} monocytes in their peripheral blood, a cell type linked to vessel occlusion and vascular disease (20–22). To determine if inactivation of *Nf1* in myeloid cells would be sufficient to mobilize Ly6C^{hi} cells, which are the murine

correlate of human pro-inflammatory monocytes (23,24), we generated mice that were heterozygous or homozygous for *Nf1* in myeloid cells. Briefly, *Nf1*^{fl/fl} mice, which contain conditional *Nf1* alleles susceptible to *Cre*-mediated recombination, were intercrossed with *LysMcre* mice to generate *Nf1*^{fl/+}; *LysMcre* (heterozygous) or *Nf1*^{fl/fl}; *LysMcre* (homozygous) progeny.

Similar to NF1 patients, *Nf1*^{+/-} and *Nf1*^{fl/+}; *LysMcre* mice had increased absolute numbers of circulating Ly6C^{hi} cells in their peripheral blood when compared with WT controls at baseline as assessed by polychromatic flow cytometry (Fig. 1A and B). Genetic ablation of the second *Nf1* allele in myeloid cells alone (*Nf1*^{fl/fl}; *LysMcre*) resulted in a 2.5-fold increase in circulating Ly6C^{hi} monocytes when compared with mice harboring a single *Nf1* allele in myeloid cells (Fig. 1B). Peripheral blood neutrophil populations were unchanged in *Nf1*^{+/-}, *Nf1*^{fl/+}; *LysMcre* or *Nf1*^{fl/fl}; *LysMcre* when compared with WT mice (data not shown). These data provide genetic evidence that mobilization of this discrete monocyte population is cell autonomous and not dependent on growth factor secretion by other cell types *in vivo*.

Heterozygous or homozygous inactivation of *Nf1* in myeloid cells is sufficient for enhanced neointima formation following arterial injury *in vivo*

To determine the effect of heterozygous inactivation of *Nf1* in myeloid cells on neointima formation after arterial injury, *Nf1*^{fl/+}; *LysMcre* mice underwent carotid artery ligation and analysis for neointima formation along with WT and *Nf1*^{+/-} experimental controls. Carotid artery ligation is a well-established model that induces neointima formation through alterations in arterial hemodynamics (25).

Histologic examination of Van Gieson-stained sections from the uninjured carotid artery of each experimental group were morphologically similar and showed no evidence of neointima formation (Fig. 2A–C). However, in response to carotid artery ligation, *Nf1*^{+/-} and *Nf1*^{fl/+}; *LysMcre* mice had a 2-fold increase in both neointima area (Fig. 2A and B) and intima/media (I/M) ratio (Fig. 2C) when compared with WT mice. Media area was similar in each genotype, indicating that cell proliferation and accumulation is limited to the intimal layer (data not shown). There was no statistical difference in neointima size or I/M ratios between *Nf1*^{+/-} and *Nf1*^{fl/+}; *LysMcre* mice (Fig. 2A–C). Consistent with prior experiments, macrophages are the predominant hematopoietic cell within the mature neointima of *Nf1*^{fl/+}; *LysMcre* mice as identified by anti-Mac3 (Fig. 2D). Neutrophils, as identified by anti-NIMP-R14 antibody, were recruited to the evolving neointima by day 7 and were absent from the mature neointimas (day 28) observed in all three genotypes (data not shown).

Lysozyme M is expressed in monocytes and granulocytes (26) and, importantly, is not expressed in vascular wall cells (data not shown). To assess the contribution of neurofibromin-deficient neutrophils and monocytes/macrophages, we examined arterial cross sections of the evolving neointima in *Nf1*^{+/-} and WT mice 7 days after arterial injury. Cross sections of *Nf1*^{+/-} and WT carotid arteries did not significantly differ in the number of infiltrating myeloid cells to the neointima of *Nf1*^{+/-} and WT mice as determined by staining for

Figure 1. Ly6C^{hi} monocytes are mobilized in a cell-autonomous manner. (A) Representative gating strategy: within the CD115-positive population (red box), monocytes are distinguished by positive staining for Ly6C (x-axis) and negative staining for CD11c (y-axis). Pro-inflammatory Ly6C^{hi} monocytes are identified within the green box for representative WT, *Nf1*^{+/−};LysMcre and *Nf1*^{fl/fl};LysMcre mice. (B) Absolute number of Ly6C^{hi} monocytes in peripheral blood of WT, *Nf1*^{+/−}, *Nf1*^{fl/fl};LysMcre and *Nf1*^{fl/fl};LysMcre. Data represent mean percentage ± SEM, *n* = 10. **P* < 0.02 for WT versus *Nf1*^{+/−} and *Nf1*^{fl/fl};LysMcre. ***P* < 0.0001 for WT, *Nf1*^{+/−} and *Nf1*^{fl/fl};LysMcre versus *Nf1*^{fl/fl};LysMcre. Analysis by one-way ANOVA with Tukey's post-hoc test. There was no statistical difference between *Nf1*^{+/−} and *Nf1*^{fl/fl};LysMcre mice.

macrophages [3.2 versus 3.5 per high power field (HPF), respectively] or neutrophils (5.3 versus 4.8 per HPF, respectively) (Fig. 3).

Finally, homozygous deletion of *Nf1* in myeloid cells alone significantly increased arterial occlusion (Fig. 4A). As a result, homozygous deletion of *Nf1* in myeloid cells resulted in a significant increase in the percentage of lumen stenosis as compared to *Nf1* heterozygous and WT mice, thus providing

genetic evidence that ablation of both *Nf1* alleles in myeloid cells further enhances neointima size *in vivo* (Fig. 4B). These data demonstrate that heterozygous inactivation of *Nf1* in myeloid cells alone reproduces the vascular phenotype observed in *Nf1*^{+/−} mice and identify *Nf1*^{+/−} myeloid cells as the critical hematopoietic cell effector of NF1 vasocclusive disease.

Rosuvastatin reduces the enhanced function of neurofibromin-deficient macrophages and VSMCs to growth factors *in vitro*

Neurofibromin-deficient myeloid progenitor cells and their differentiated progeny demonstrate increased proliferation, migration and survival in response to various growth factors implicated in neointima formation (15,16,27). Rosuvastatin attenuates monocyte recruitment and adhesion to the vascular wall, reduces inflammatory cytokine and MMP production and inhibits neointima formation following arterial injury *in vitro* and *in vivo* (28–30). Based on these observations and the predominance of *Nf1*^{+/−} macrophages within *Nf1*^{+/−} neointimas at day 28 following arterial injury, we tested whether rosuvastatin would abrogate the enhanced proliferation, migration and adhesion observed in *Nf1*^{+/−} macrophages *in vitro* in response to M-CSF, which is a growth factor previously linked to vascular inflammation and arterial wall remodeling (27). Incubation of *Nf1*^{+/−} macrophages with rosuvastatin prior to M-CSF stimulation diminished *Nf1*^{+/−} macrophage proliferation, migration and adhesion when compared with control (Fig. 5A–C).

VSMC proliferation, which is partially induced by growth factor secretion from infiltrating macrophages, within the intimal layer of the arterial wall is the central mechanism of *Nf1*^{+/−} arterial stenosis. We previously demonstrated that *Nf1*^{+/−} VSMCs have increased proliferation to various growth factors and *Nf1*^{+/−} macrophages both *in vitro* and *in vivo* (12,15). Therefore, we tested whether rosuvastatin would abrogate the increased proliferation of *Nf1*^{+/−} VSMCs in response to TNFα, which is a cytokine secreted by circulating macrophages, induces VSMC proliferation through Ras-Erk signaling and is critical for neointima formation (31–33). *Nf1*^{+/−} VSMCs demonstrated increased proliferation when compared with WT controls in response to multiple doses of TNFα (Fig. 6A). However, 5 and 10 μM rosuvastatin reduced *Nf1*^{+/−} VSMC proliferation to WT levels without growth factor stimulation (Fig. 6B). Collectively, these data demonstrate that rosuvastatin inhibits multiple cell functions of both *Nf1*^{+/−} macrophages and VSMCs that are essential for the initiation and progression of neointima formation.

Administration of rosuvastatin inhibits neointima formation in *Nf1*^{+/−} mice, following arterial injury *in vivo*

The pleiotropic effects of statins are well described and function independent of their lipid-lowering properties (34–36). Although specific mechanisms for their anti-inflammatory effects remain elusive, several studies provide evidence that statins reduce acute phase reactants and inflammatory cytokines linked to cardiovascular disease and arterial remodeling

Figure 2. Heterozygous inactivation of *Nf1* in myeloid cells is sufficient for enhanced neointima formation. (A) Representative Van Gieson-stained cross sections of uninjured and injured carotid arteries from WT, *Nf1*^{+/-} and *Nf1*^{fl/+}; *LysMcre* mice. Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified in the far-right panels. Scale bars: 100 μ m. (B and C) Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from WT, *Nf1*^{+/-} and *Nf1*^{fl/+}; *LysMcre* mice. Data represent the mean neointima area of three arterial cross sections (400, 800 and 1200 μ m proximal to the ligation) $\pm$ SEM, $n = 10-12$. * $P < 0.01$ for WT uninjured versus WT injured, *Nf1*^{+/-} uninjured versus *Nf1*^{+/-} injured and *Nf1*^{fl/+}; *LysMcre* uninjured versus *Nf1*^{fl/+}; *LysMcre* injured. ** $P < 0.01$ for WT injured versus *Nf1*^{+/-} injured and *Nf1*^{fl/+}; *LysMcre* injured. Analysis by one-way ANOVA. There was no statistical difference between *Nf1*^{+/-} and *Nf1*^{fl/+}; *LysMcre* injured. (D) Representative photomicrograph of injured carotid artery cross section from *Nf1*^{fl/+}; *LysMcre* mice stained with anti-Mac3 antibody (brown) and counterstained with hematoxylin (blue). Black arrows indicate neointima boundaries. Black arrowheads represent positive Mac3 staining. Black box identifies area of injured artery that is magnified in the far-right panel. Scale bars: 100 μ m.

Figure 3. Macrophages and neutrophils are recruited to the evolving neointima by 7 days post-injury. Representative photomicrographs from injured carotid arteries from WT (top panels) and *Nf1*^{+/-} (bottom panels) mice demonstrating macrophage (left column) and neutrophil (right column) infiltration into the neointima 7 days after arterial ligation. Black arrows indicate neointima boundaries. Black arrowheads represent positive macrophage (anti-Mac3) and neutrophil (anti-NIMP-R14) staining. Black box identifies area of injured artery that is magnified below. Scale bars: 100 μ m. For each carotid artery, Mac-3-positive and NIMP-R14-positive neointimal cells were counted separately in five distinct 40 $\times$ images at 400, 800 and 1200 μ m proximal to the bifurcation, and average cells per HPF were calculated ($n = 5$ per group). There was no difference in number of macrophages or neutrophils per HPF in the intimal layer between WT and *Nf1*^{+/-} mice.

(34,35). Furthermore, statin use in experimental animal models reduces neointima formation, following vascular injury (30,36,37).

Given these observations, we tested whether administration of oral rosuvastatin would inhibit neointima formation in *Nf1*^{+/-} mice following mechanical arterial injury. Utilizing the carotid artery ligation model previously described,

Nf1^{+/-} and WT mice were administered either phosphate buffered saline (PBS) or 10 mg/kg rosuvastatin daily for 7 days prior to vascular injury and continued on daily dosing through tissue harvest at 28 days post-injury. In response to carotid artery injury, rosuvastatin treatment greatly reduced neointima formation in *Nf1*^{+/-} mice when compared with PBS controls (Fig. 7A–C). Morphometric analysis of

Figure 4. Arterial occlusion and stenosis is regulated by neurofibromin. (A) Representative Van Gieson-stained cross section of uninjured and injured carotid arteries from *Nf1*^{fl/fl}; *LysMcre* mice. Black arrows indicate neointima boundaries. Scale bar: 100 μ m. (B) Quantification of lumen stenosis of injured carotid arteries from WT, *Nf1*^{+/-}, *Nf1*^{fl/fl}; *LysMcre* and *Nf1*^{fl/fl}; *LysMcre* arteries. Data represent the mean percentage of lumen stenosis of three arterial cross sections (400, 800 and 1200 μ m distal to the ligation) $\pm$ SEM, $n = 10$ – 12 . * $P < 0.01$ for WT versus *Nf1*^{+/-} and *Nf1*^{fl/fl}; *LysMcre*. ** $P < 0.01$ for WT, *Nf1*^{+/-}, *Nf1*^{fl/fl}; *LysMcre* mice versus *Nf1*^{fl/fl}; *LysMcre*. Analysis by one-way ANOVA. There was no statistical difference between *Nf1*^{+/-} and *Nf1*^{fl/fl}; *LysMcre*.

rosuvastatin-treated *Nf1*^{+/-} mice demonstrated a 40% reduction in neointima area and a 50% reduction in I/M ratio when compared with PBS-treated *Nf1*^{+/-} mice in response to arterial injury (Fig 7B and C). No significant difference in either intima area or I/M ratio in response to injury was observed in WT mice when comparing treatment groups. Importantly, rosuvastatin did not alter total cholesterol, triglyceride levels or circulating peripheral blood hematopoietic and progenitor cell populations in either genotype (data now shown).

DISCUSSION

Cardiovascular disease is an important contributor to excess morbidity and mortality in NF1 patients (4). Currently, there are no recommendations for screening NF1 patients for cardiovascular disease and specific treatment options are limited. Thus, a true estimate of the disease burden and natural pathology of NF1-related vasculopathy remains elusive.

In this study, we demonstrate that inactivation of *Nf1* in myeloid cells alone is sufficient to mobilize a discrete population of inflammatory murine monocytes and enhance neointima formation when compared with WT mice. Specifically, *Nf1*^{fl/fl}; *LysMcre* mice form neointimas similar to *Nf1*^{+/-} mice with VSMC accumulation limited to the intimal layer, which completely replicates the phenotype previously observed in *Nf1*^{+/-} mice. Although germline loss of both *Nf1* alleles in myeloid cells has not been demonstrated in NF1 vascular disease, loss of the second *Nf1* allele in mice further mobilizes Ly6C^{hi} monocytes and amplifies the degree of arterial stenosis providing genetic evidence that *Nf1* is a critical regulator of myeloid cell function and

Figure 5. Rosuvastatin reduces proliferation, migration and adhesion in *Nf1*^{+/-} macrophages. (A) WT (white bars) and *Nf1*^{+/-} (black bars) macrophage proliferation in response to stimulation with M-CSF (20 ng/ml) in the presence of rosuvastatin, where indicated. Data represent thymidine incorporation reported as mean counts per minute (cpm) $\pm$ SEM, $n = 6$. * $P < 0.001$ for WT versus *Nf1*^{+/-} macrophages stimulated with M-CSF. ** $P < 0.001$ for WT macrophages without growth factor versus WT macrophages incubated with M-CSF and *Nf1*^{+/-} macrophages without growth factor versus *Nf1*^{+/-} macrophages incubated with M-CSF. # $P < 0.01$ for *Nf1*^{+/-} macrophages stimulated with M-CSF versus *Nf1*^{+/-} macrophages stimulated with M-CSF in the presence of 10 and 20 μ M rosuvastatin. Analysis was performed by one-way ANOVA. (B) WT (white bars) and *Nf1*^{+/-} (black bars) macrophage migration in response to stimulation with M-CSF (100 ng/ml) in the presence of rosuvastatin, where indicated. Data represent average number of migrated cells per HPF $\pm$ SEM, $n = 6$. * $P < 0.01$ for WT macrophages versus *Nf1*^{+/-} macrophages in the absence and presence of M-CSF. ** $P < 0.001$ for WT macrophages without M-CSF versus WT macrophages incubated with M-CSF and *Nf1*^{+/-} macrophages without M-CSF versus *Nf1*^{+/-} macrophages incubated with M-CSF. # $P < 0.001$ for WT macrophages stimulated with M-CSF versus WT macrophages stimulated with M-CSF in the presence of 10 μ M rosuvastatin and *Nf1*^{+/-} macrophages stimulated with M-CSF in the presence of 10 μ M rosuvastatin. Analysis by one-way ANOVA. (C) WT (white bars) and *Nf1*^{+/-} (black bars) macrophage adhesion to fibronectin. Data represent mean optical density $\pm$ SEM, $n = 4$. * $P < 0.01$ for WT versus *Nf1*^{+/-} macrophages; ** $P < 0.01$ for *Nf1*^{+/-} macrophages versus *Nf1*^{+/-} macrophages incubated with 10 μ M rosuvastatin at 15 and 30 min. Analysis by one-way ANOVA.

Figure 6. *Nf1*^{+/-} VSMCs have increased response to TNFα that is abrogated by incubation with rosuvastatin. (A) WT (white bars) and *Nf1*^{+/-} (black bars) VSMC proliferation in response to stimulation with TNFα. Data represent thymidine incorporation reported as mean cpm ± SEM, *n* = 5. **P* < 0.001 for WT versus *Nf1*^{+/-} VSMCs stimulated with indicated concentrations of TNFα. ***P* < 0.01 for WT and *Nf1*^{+/-} VSMCs versus WT and *Nf1*^{+/-} VSMCs stimulated with indicated concentrations of TNFα. Analysis by one-way ANOVA with Tukey's post-hoc test. (B) WT (white bars) and *Nf1*^{+/-} (black bars) VSMC proliferation in response to stimulation with TNFα in the presence of rosuvastatin, where indicated. Data represent thymidine incorporation reported as mean cpm ± SEM, *n* = 6. **P* < 0.001 for WT VSMC stimulated with TNFα versus *Nf1*^{+/-} VSMCs stimulated with TNFα. ***P* < 0.001 for WT and *Nf1*^{+/-} VSMCs versus WT and *Nf1*^{+/-} VSMCs stimulated with TNFα. #*P* < 0.001 for *Nf1*^{+/-} VSMCs stimulated with TNFα versus *Nf1*^{+/-} VSMCs stimulated with TNFα in the presence of 5 and 10 μM rosuvastatin. Analysis by one-way ANOVA.

neointima formation. Using isolated primary cells, we show that *Nf1*^{+/-} macrophages have increased proliferation, migration and adhesion in response to M-CSF, which is significantly reduced when cells are preincubated with rosuvastatin. Furthermore, we show that preincubation of TNFα-stimulated *Nf1*^{+/-} VSMCs with rosuvastatin reduces their proliferation to levels observed in unstimulated WT VSMCs. This is an important observation because VSMC proliferation is a critical cellular mechanism of arterial stenosis and neointima formation. Finally, based on these *in vitro* results, we show that once daily administration of oral rosuvastatin is sufficient to reduce neointima formation in *Nf1*^{+/-} mice after carotid artery ligation *in vivo*.

Neurofibromin functions as a negative regulator of Ras activity in myeloid cells and renders them hypersensitive to growth factors (1,38). Specifically, granulocyte-macrophage progenitors, mast cells and osteoclasts are hypersensitive to multiple growth factors predisposing some NF1 patients to the development of juvenile myelomonocytic leukemia, plexiform neurofibromas and bone disorders (16,38,39). Thus, the finding that neurofibromin is a critical regulator of monocyte/macrophage function and that loss of neurofibromin in myeloid cells directly contributes to *Nf1*^{+/-} neointima

formation is consistent with other manifestations of NF1. Furthermore, asymptomatic NF1 patients have increased circulating levels of pro-inflammatory monocytes and cytokines linked to cardiovascular disease supporting the hypothesis that neurofibromin is a critical regulator of vascular homeostasis and that heterozygous inactivation of NF1 leads to vascular inflammation (15). In support of this hypothesis, murine Ly6C^{hi} monocytes, an important pro-inflammatory monocyte population, are independently mobilized in mice with heterozygous and homozygous deletion of *Nf1* in myeloid cells, which is similar to *Nf1*^{+/-} mice. Importantly, Ly6C^{hi} monocytes express high cell surface levels of the CCR2 receptor and are actively recruited to sites of inflammation and differentiate into macrophages and dendritic cells (40,41). Thus, the finding that Ly6C^{hi} monocytes are increased in *Nf1*^{+/-} mice and mobilized in a cell-autonomous manner indicates that CCR2 signaling may be important in *Nf1*^{+/-} neointima formation. Ongoing studies are currently investigating this hypothesis in an attempt to further identify therapeutic targets to treat NF1 vascular disease because pharmaceutical agents are already available to inhibit the CCR2 signaling axis.

Emerging evidence suggests that Ras signaling tightly regulates vascular wall homeostasis at rest and in response to growth factor stimulation, and perturbations of Ras signaling can result in a variety of cardiovascular disease manifestations. In fact, patients with diverse genetic disorders that affect Ras signaling, which are now termed the 'Rasopathies,' display various degrees of cardiovascular disease.

Recent animal studies also demonstrate that amplification of Ras signaling significantly enhances neointima formation, whereas inhibition of the canonical Ras-Mek-Erk pathway reduces neointima formation and VSMC proliferation and migration *in vivo* (42,43). In support of the hypothesis that enhanced Ras signaling augments neointima formation, previous histologic examination of arterial cross sections from *Nf1*^{+/-} neointimas showed increased Erk phosphorylation when compared with WT controls (14). Furthermore, treatment of *Nf1*^{+/-} mice with Gleevec, an important inhibitor of the PDGF-BB-Ras-Erk signaling pathway, significantly reduced neointima formation and Erk phosphorylation in *Nf1* heterozygous mice (14). Although mounting evidence suggests that Ras-Erk signaling actively participates in hematopoietic cell recruitment to the injured artery, regulates proliferation and migration of VSMCs and augments remodeling of the vascular wall, specific drug-targeted inhibition of the Ras-Mek-Erk pathway to reduce neointima formation following mechanical injury has not been demonstrated *in vivo*. Testing whether current MEK inhibitors, which are in clinical use for other diseases, inhibit neointima formation in our NF1 mouse model of vascular disease could provide further therapeutic insights.

Statins have been used in several NF1 patient populations and animal models of NF1-related pathologies as an investigational therapeutic agent. In murine models of NF1 skeletal dysgenesis, statin use improved bone marrow mineralization and reduced Ras activation in neurofibromin-deficient osteoblasts and osteochondroprogenitors, a subset of the myeloid cell lineage (16,44). Interestingly, statin use improves cognitive function in *Nf1* heterozygous mice and regulates functional connectivity in the brain of NF1 patients, although it failed

Figure 7. Rosuvastatin reduces neointima formation in *Nf1*^{+/-} mice. (A) Representative Van Gieson-stained cross sections of uninjured and injured carotid arteries from rosuvastatin-treated WT and *Nf1*^{+/-} mice. Representative photomicrographs of H&E-stained carotid arteries from WT (top panels) and *Nf1*^{+/-} (bottom panels) mice 28 days, following no injury (left panels), injury and PBS treatment (middle panels) or injury and rosuvastatin treatment (right panels). Black arrows indicate neointima boundaries. Black boxes identify area of injured artery that is magnified below. Scale bars: 100 μ m. (B and C) Quantification of neointima area (B) and I/M ratio (C) of injured carotid arteries from PBS and rosuvastatin-treated WT and *Nf1*^{+/-} mice. Data represent the mean neointima area of three arterial cross sections (400, 800 and 1200 μ m distal to the ligation) $\pm$ SEM, $n = 10-12$. * $P < 0.01$ for WT uninjured versus WT injured with PBS treatment and *Nf1*^{+/-} uninjured versus *Nf1*^{+/-} injured with PBS treatment. ** $P < 0.01$ for WT injured with PBS treatment versus *Nf1*^{+/-} injured with PBS treatment. # $P < 0.01$ for *Nf1*^{+/-} injured with PBS treatment versus *Nf1*^{+/-} injured with rosuvastatin treatment. Analysis by one-way ANOVA.

to produce significant improvement in cognitive functioning in a randomized clinical trial, which demonstrates the complex functionality of statins as a therapeutic agent in treating non-neoplastic manifestations of NF1 (45).

Statins are an important clinical therapeutic with wide-ranging pleiotropic effects that appear to extend beyond their lipid-lowering profile and may protect patients with subtle cardiovascular risk factors from developing overt

cardiovascular disease (34–36,46). Although these effects may be generalizable to the entire statin group, human studies assessing the effect of rosuvastatin demonstrate a reduction in major cardiovascular events in asymptomatic patients with elevated inflammatory markers without hyperlipidemia (18,19). These studies suggest that chronic inflammation contributes to cardiovascular disease and that rosuvastatin may possess anti-inflammatory properties through inhibition of various signaling pathways in myeloid cells that control the secretion of inflammatory mediators, though the mechanism for these effects is not completely understood (18,19). Relevant to the current study, previous murine studies of primary cells and neointima formation demonstrated that rosuvastatin reduced VSMC proliferation and neointima formation *in vitro* and *in vivo* (17,30). More importantly, emerging evidence from murine models and human clinical trials suggests that statins, particularly atorvastatin and rosuvastatin, suppress the production of reactive oxygen species and cytokines that contribute to vascular inflammation (28,47,48). Of note, in contrast to prior studies, rosuvastatin (10 mg/kg/day) failed to diminish neointima formation significantly in WT mice. However, prior studies in mouse and rat neointima models showed significant neointima reduction at a higher daily dosing range of 20–40 mg/kg/day (30,49,50), which could potentially explain differences in our experimental results. Furthermore, statins display divergent effects on important signaling cascades, including Jak-STAT, Ras-Mek-Erk and PI-3K, which contribute to the various observed cardiovascular effects (51,52). Thus, the increased response of *NfI*^{+/-} myeloid cells to growth factors likely renders them more sensitive to alterations in transcellular signaling pathways and more responsive to lower doses of rosuvastatin *in vivo*. Given the pleiotropic effects of rosuvastatin, understanding the precise molecular mechanism of how this therapy diminishes neointima formation in *NfI*^{+/-} mice will provide more insights into the molecular mechanism of NF1 vascular disease and will potentially reveal new therapeutic options.

In this report, we showed that heterozygous inactivation of *NfI* in myeloid cells is sufficient to enhance neointima formation in *NfI*^{+/-} mice. This is the first report demonstrating that loss of the *NfI* allele in a single cell lineage is sufficient to cause cardiovascular disease and provides potential important insights into the development of cardiovascular disease in NF1 patients. Consistent with our previous observations in NF1 patients, we also observed a cell autonomous mobilization of inflammatory monocytes in *NfI*^{+/-} and *NfI*^{fl/+}; *LysMcre* mice, which supports our overarching hypothesis that NF1 patients experience chronic inflammation that predisposes them to cardiovascular disease. Finally, we show that rosuvastatin reduces neointima formation in *NfI*^{+/-} mice when compared with placebo, which may be a therapeutic option for the prevention and/or treatment of NF1 patients predisposed to cardiovascular disease.

MATERIALS AND METHODS

Animals

Protocols were approved by the Indiana University Laboratory Animal Research Center. *NfI*^{+/-} mice were obtained

from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA, USA) and backcrossed for 13 generations into the C57BL/6J strain. *NfI*^{fl/fl} mice were obtained from Luis Parada (University of Texas Southwestern Medical Center, Dallas, TX, USA) and backcrossed for 13 generations into the 129SvJ strain (53). *LysMcre* (stock 4781) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on C57BL/6 strain. *NfI*^{fl/fl} mice were crossed with *LysMcre* mice to generate C57BL/6 × 129SvJ pups. *NfI*^{fl/+}; *LysMcre* (heterozygous loss of *NfI* in myeloid cells only) and *NfI*^{fl/fl}; *LysMcre* (homozygous loss of *NfI* in myeloid cells only) mice were used for experiments. *LysM* is expressed in neutrophils and macrophages (54). *LysM* is expressed in monocytes (80% efficiency) and granulocytes (100% efficiency) (26). *Cre*-mediated recombination was confirmed by PCR as previously described (53). Crossing *NfI*^{fl/+}; 129SvJ mice with *NfI*^{+/-} C57BL/6 mice generated *NfI* heterozygous and WT controls for experiments. Male mice, between 12 and 15 weeks of age, were used for experiments.

Carotid artery ligation

Carotid artery injury was induced by ligation of the right common carotid artery as previously 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 via IP injection following the procedure and recovered for 7 days or 28 days without complication.

Rosuvastatin administration

When stated, rosuvastatin (10 mg/kg/day; AstraZeneca, London, UK) was administered once per day via oral gavage. Rosuvastatin treatment commenced 7 days prior to carotid artery injury and continued through tissue harvest at 28 days post-injury. PBS was given in an equivalent volume as a control.

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 (National Institute of Health, Bethesda, MD, USA). 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 aorta were excluded from analysis. 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. Percentage of lumen stenosis was calculated as intima area divided by IEL area and reported as a percentage value. Finally, due to variability in arterial remodeling over the length of the injured artery, photomicrographs in each of the figures demonstrate representative

arterial cross sections between 800 and 1200 μm proximal to the carotid bifurcation.

Histopathology and immunohistochemistry

Twenty-eight days after ligation, whole ligated and contralateral uninjured carotid arteries were harvested from mice as previously described (14,15). Briefly, mice were anesthetized with an isoflurane (2%)/oxygen (98%) mixture and were perfused fixed at constant pressure (100 mmHg) with 10 ml of 125 mM adenosine plus 0.0125 mM sodium nitroprusside for 5 min followed by Z-fix solution (Anatech, Battle Creek, MI, USA). Under a dissecting scope, the injured and uninjured common carotid arteries were excised, then fixed overnight at 4°C in Z-fix solution and paraffin embedded. Serial 7 μm arterial cross sections were made at 200 μm intervals across the length of the carotid artery. Van Gieson staining was performed according to standard methods (EMS, Hatfield, PA, USA).

For immunohistochemistry, sections were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol, following antigen retrieval in Antigen Unmasking Solution (Vector Laboratories, Berlingame, CA, USA) at 95°C. Sections were blocked with Protein Block (Dako, Denmark) for 1 h and were incubated with anti-Mac3 (1:50; BD Biosciences, San Jose, CA, USA), anti-NIMP-R14 (1:50; Abcam, Cambridge, MA, USA) or anti-Ki67 (1:25; Dako) primary antibodies. Sections were incubated with appropriate biotinylated secondary antibody (Vector Laboratories) and visualized by 3,3'-diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin. Sections were examined, and images of sections were collected using a Zeiss Axioskop microscope (Carl Zeiss, Chester, VA, USA) with a 20 $\times$ or 40 $\times$ CP-ACHROMAT/0.12NA objective. Images were acquired using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Polychromatic flow cytometry

Blood samples were obtained via tail vein at days 0, 7 and 28, following injury and collected into EDTA tubes (BD Biosciences, San Jose, CA, USA). Peripheral blood was collected separately for white blood cell count using the Hemavet 950 (Drew Scientific, Dallas, TX, USA). Red blood cell lysis was performed (Qiagen, Germantown, MD, USA), and cells were washed in 2% fetal bovine serum (FBS) in PBS. Following centrifugation, each sample was resuspended in 2% FBS in PBS and incubated with murine Fc Blocking Reagent (Miltenyi Biotec, Cologne, Germany) for 10 min on ice. Cell aliquots were incubated with either CD 115-PE (eBioscience, San Diego, CA, USA), CD11c-PERCPy5.5 (BD Biosciences), CD11b-APC-Cy7 (BD Biosciences), Ly6C-PE-Cy7 (BD Biosciences), F4/80-APC (Serotech, Oxford, UK), F7/4-FITC (Serotech), TER119-Pac Blue (eBioscience) and LIVE/DEAD violet-fixable dead cell stain (Invitrogen, Grand Island, NY, USA) or separately CD34-FITC (BD Biosciences), CD117-APC (BD Biosciences), Fc ϵ R1a-PE (eBioscience), CD19-PECy7 (BD Biosciences), CD3-APCCy7 (BD Biosciences), TER-119-Pac Blue (eBioscience) and LIVE/DEAD violet-fixable dead cell stain (Invitrogen) for 30 min and

resuspended in 2% FBS in PBS. Antibodies were titrated for optimal staining, and 'fluorescence-minus-one' gating controls were performed to determine true positive and negative events. Stained 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 100 000 events were collected for samples. Data were collected uncompensated and analyzed using FlowJo software version 8.7.3 (Tree Star). After excluding red blood cells, apoptotic and dead cells, total Ly6C^{hi} monocytes were calculated by multiplying the percentage of Ly6C^{hi} cells per total live cells by the peripheral white blood cell count for each individual sample.

Isolation of bone marrow-derived macrophages and characterization

Bone marrow-derived macrophage isolation was performed as described (15). Macrophage proliferation, migration and adhesion assays were performed as described (15). Where indicated, cells were pretreated with the indicated concentration of rosuvastatin or vehicle for 1 h.

VSMC isolation and proliferation

VSMC isolation and subsequent proliferation assays were performed as described (12). Where indicated, cells were pretreated with the indicated concentration of rosuvastatin or vehicle for 1 h.

Statistical analysis

All values are presented as mean $\pm$ SEM. Circulating monocyte frequency was assessed by one-way ANOVA. Intima area and I/M ratio analysis was assessed by one-way ANOVA with a Tukey's post hoc test using GraphPad Prism version 5.0d (GraphPad Software, San Diego, CA, USA). $P < 0.05$ were considered significant.

Conflict of Interest statement. None declared.

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