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Early Administration of Imatinib Mesylate Reduces Plexiform Neurofibroma Tumor Burden with Durable Results after Drug Discontinuation in a Mouse Model of Neurofibromatosis Type 1

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Abstract

BACKGROUND—Neurofibromatosis type 1 (NF1) is a common genetic disorder characterized by plexiform neurofibromas (pNF), which are thought to be congenital tumors that arise *in utero* and enlarge throughout life. Genetic studies in murine models delineated an indispensable role for the stem cell factor (SCF)/c-kit pathway in pNF initiation and progression. A subsequent phase 2 clinical trial using imatinib mesylate to inhibit SCF/c-kit demonstrated tumor shrinkage in a subset of pre-existing pNF, however imatinib's role on preventing pNF development has yet to be explored.

PROCEDURE—We evaluated the effect of imatinib dosed at 10–100 mg/kg/day for 12 weeks to 1-month old *Nf1*$^{flox/flox}$*;PostnCre(+)* mice, prior to onset of pNF formation. To determine durability of response, we then monitored for pNF growth at later time points, comparing imatinib to vehicle treated mice. We assessed gross and histopathological analysis of tumor burden.

RESULTS—Imatinib administered preventatively led to a significant decrease in pNF number, even at doses as low as 10 mg/kg/day. Tumor development continued to be significantly inhibited after cessation of imatinib dosed at 50 and 100 mg/kg/day. In the cohort of treated mice that underwent prolonged follow-up, the size of residual tumors was significantly reduced as compared to age-matched littermates that received vehicle control.

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The authors have no conflicts of interest to report.

Data Availability

The data that support the findings of this study are available from the corresponding author, DWC, upon reasonable request.

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CONCLUSIONS—Early administration of imatinib inhibits pNF genesis *in vivo* and effects are sustained after discontinuation of therapy. These findings may guide clinical use of imatinib in young NF1 patients prior to substantial development of pNF.

Keywords

imatinib mesylate; plexiform neurofibroma; NF1; preventative therapy

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant genetic disorder affecting about 1 in 3000 individuals worldwide [1]. Caused by heterozygous inactivating mutations in the *NF1* tumor suppressor gene that encodes for neurofibromin, NF1 is characterized by a variety of clinical presentations including peripheral nervous system tumors. Plexiform neurofibromas (pNF) develop in 30–50% of patients with NF1 [2, 3] and are composed of proliferating neoplastic Schwann cells within a heterogeneous microenvironment of fibroblasts, macrophages, mast cells, perineurial cells, and secreted collagen [4–6]. The majority of pNF are detected in early childhood and progressively enlarge throughout life, with the highest growth rate in younger children [7–9]. These tumors are highly morbid via pain, motor, and sensory dysfunction related to involvement of nerve fascicles, can compress vital structures, and can lead to substantial visible disfigurement. Finally, the major cause of mortality in people with NF1 is malignant peripheral nerve sheath tumors (MPNSTs), which occur in approximately 10% of people with NF1 and arise from pNF burden [10].

Plexiform neurofibromas are thought to originate from Schwann cells or their early precursors [4, 6, 11–14], where aberrant stem cell factor (SCF)/c-kit signaling emanating from the tumor microenvironment promotes the initiation and growth of pNF lesions in genetically engineered mouse models (GEMM) [5]. Accordingly, imatinib mesylate, a potent inhibitor of c-kit, along with PDGFR and c-abl tyrosine kinases, induced the first objective clinical response seen in a therapeutic phase 2 trial in NF1 patients with pNF [15]. Over the past decade, further knowledge that neurofibromin loss results in hyperactivation of the Ras signaling cascade and other signal transduction networks has led to a variety of molecular targeted approaches and clinical trials for NF1-associated pNF [16–18], including inhibition of MEK [19], VEGF [20], and mTOR [21]. More recently, the role of nerve injury and subsequent inflammation in neurofibromagenesis has been evaluated, with the suggestion dual inhibition of both mast cells and macrophages may be effective [22]. Preclinical studies have also assessed the preventative role of MEK inhibition, which did not inhibit pNF development but did alter tumor size [23]. Despite these therapeutic advances, we are still faced with the challenge that not all existing pNF respond to targeted therapy and tumors often regrow when therapy is discontinued.

Capitalizing on previous observations that younger people with pNF that were smaller in total volume and located in the head, neck, and airway region had excellent tumor responses with imatinib [5,15], which is FDA approved for use in infants with leukemia, we evaluated the potential of imatinib for preventing the formation of pNF by blocking mast-cell recruitment to the microenvironment during the nascent stages of tumor development in

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genetically engineered *Nf1*$^{flox/flox}$; *PostnCre(+)* mice. Beginning at 1 month of life, preceding the earliest histological evidence of tumor formation, we determined that administering imatinib was sufficient to prevent pNF initiation. Further, when followed for 20 weeks after cessation of imatinib, the number and size of residual tumors were significantly reduced as compared to littermates that received vehicle control. Collectively, these data demonstrate a proof of concept that imatinib may be effective as a preventative therapy for pNF with durable outcomes.

MATERIAL AND METHODS

Experimental Animals

The genetically engineered *Nf1*$^{flox/flox}$*;PostnCre(+)* mice have been previously described [24, 25]. Animal care and experiments were conducted according to the guidelines established by the Indiana University Animal Care and Use Committee. Progeny from these crosses were genotyped by polymerase chain reaction as described previously [24].

In vivo Experimental Design and Drug Treatment Protocols

Weight-based doses of either imatinib or the vehicle control, phosphate buffered saline, were administered by daily oral gavage to cohorts of *Nf1*$^{flox/flox}$*;PostnCre(+)* mice that were age and sex-matched as best as possible (details of sex in Supplemental Table 1). To determine the biological effect of varying doses of imatinib on preventing the development of pNFs, doses of 10, 25, 50, and 100 mg/kg of imatinib or vehicle control were administered daily for 12 weeks to cohorts of *Nf1*$^{flox/flox}$*;PostnCre(+)* mice beginning at approximately 1 month of life, prior to the onset of tumors (Supplemental Fig. 1). At the end of 12 weeks of therapy, the animals were euthanized for gross and histopathological analysis of tumor burden. Of note, by four months of life, *Nf1*$^{flox/flox}$*;PostnCre(+)* mice develop plexiform neuorfibromas in multiple peripheral nerves with 100% penetrance [24, 25]. To determine whether early administration of imatinib results in stable tumor inhibition, 1 month old *Nf1*$^{flox/flox}$*;PostnCre(+)* mice were treated with 50 and 100 mg/kg/day of imatinib or vehicle control for 12 weeks. Treatment was discontinued, and the mice were then euthanized at 20 weeks following the end of drug therapy to evaluate for gross and histopathological evidence of tumor reemergence or progression.

Nerve Tree Microdissection and Measurement of Tumor Volume

Immediately postmortem, fresh tissues were harvested and mice were fixed in 10% neutral buffered formalin. The bodies were decalcified in 5% formic acid in 10% neutral buffered formalin solution. The proximal spinal nerve roots from the lumbosacral spine were dissected microscopically. The volume of proximal peripheral nerves was determined using calipers to measure the length and width of dissected tumors (or equivalent region in absence of tumor) in maximal dimension. Volume was approximated using the formula for the volume of a spheroid ($0.52 \times (\text{width})^2 \times \text{length}$) according to our previously established methodology [5].

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Histological Analysis

Microdissected nerve trees were embedded in 2% agar, processed through graded alcohols and xylenes, embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin and Masson's trichrome to examine the tumor histomorphology and collagen deposition as described previously [4]. Infiltrating mast cells were identified on toluidine blue stained sections as previously reported [4]. Whole slide images were captured with an Aperio CS2 (Leica Biosystems, Buffalo Grove, IL).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 8.0 software (GraphPad, La Jolla, CA). Analysis of variance or Student's T-tests was used to evaluate for differences between samples. *P* values less than 0.05 were considered significant with additional levels of significance as indicated.

RESULTS

Early Imatinib Mesylate Therapy Prevents Plexiform Neurofibroma Initiation

Nf1$^{flox/flox}$*;PostnCre(+)* mice develop pNFs at 2–3 months of age and consistently have multiple pNFs by 4 months of age [24, 25]. Hence, we administered 10–100 mg/kg/day of imatinib or vehicle control to 1 month old *Nf1*$^{flox/flox}$*;PostnCre(+)* mice, pre-pNF appearance (Fig. 1A). Proximal spinal nerve roots were microscopically dissected to enumerate resulting tumors after 12 weeks of treatment. At 100 mg/kg/day (the established MTD), we observed a 70% reduction in the mean number of pNF per animal. Most interestingly, this significant reduction in number of mean pNF per animal was maintained at all lower doses, including 10 mg/kg/day (10% of the MTD) ($p<0.001$) (Fig. 1B).

Sustained Plexiform Neurofibroma Growth Inhibition with Early Imatinib Mesylate Followed by Prolonged Treatment Cessation

The decrease in number of pNF following early imatinib treatment, especially at low doses, was encouraging. However, it remains unclear whether the tumor cells were eliminated or retain their proliferative potential. To address this question, cohorts of *Nf1*$^{flox/flox}$*;PostnCre(+)* mice preventatively treated with 50 and 100 mg/kg/day of imatinib or vehicle control were monitored in prolonged follow-up after cessation of treatment for 20 weeks before euthanasia (Fig. 2A). The mean number of pNF in mice previously treated with imatinib was 3.5 (at 50 mg/kg/day dosing) and 3.4 (at 100 mg/kg/day dosing) versus 9.2 tumors per mouse in age matched vehicle controls ($p<0.001$) (Fig. 2B).

Alterations in the Plexiform Neurofibroma Microenvironment Persist Following Discontinuation of Imatinib Mesylate Treatment

Correlating with gross reduction of tumor number, histologic evaluation of dorsal root ganglia and proximal nerve roots in *Nf1*$^{flox/flox}$*;PostnCre(+)* mice treated preventatively with imatinib versus vehicle treated controls showed inhibition of pNF genesis. Nerves harvested from vehicle treated mice demonstrated histological features characteristic of pNF including disrupted nerve architecture and increased cellularity, as compared to mice treated with

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imatinib 50 or 100 mg/kg/day and immediately euthanized or followed for 20 weeks after therapy (Fig. 1C, Panels 1–3; Fig. 2C, Panels 1–3). Further, there was marked reduction in fibroblast collagen deposition (Fig. 1C, Panels 4–6; Fig. 2C, Panels 4–6; Supplemental Fig. 2) and reduction in infiltrating mast cells (Fig. 1C, Panels 7–9; Fig. 2C, Panels 7–9) in nerves dissected from imatinib treated mice versus those from vehicle treated cohorts.

Sustained Attenuation of Plexiform Neurofibroma Growth Velocity with Early Imatinib Mesylate Therapy

In addition to evaluating the potential of preventing tumor initiation in young mice, we also sought to delineate the impact of early imatinib treatment on residual pNF that do form despite therapy. Measuring the size of proximal spinal nerve roots can serve as an approximation for tumor volume [5]. The volume of proximal peripheral nerves was determined in mice treated with early imatinib 0, 10, 25, 50, or 100 mg/kg/day (starting at 1 month of life) for 12 weeks followed by euthanasia at 4 months of life. A second contemporaneous cohort of mice was treated with imatinib 0, 50, or 100 mg/kg/day for 12 weeks followed by a 20-week observation period before euthanasia at approximately 9 months of life, as described above. In the mice euthanized at 4 months, there was no significant reduction in the volume of proximal nerve roots compared to mice treated with vehicle control (Fig 3A). For the older cohort of mice treated with either 50 or 100 mg/kg/day of imatinib for 12 weeks and euthanized at roughly 9 months of life, proximal nerve root volume was significantly reduced compared to vehicle treated mice (Fig 3B).

The mean proximal nerve size of the control mice in the younger cohort was 0.659 mm^3 compared to 1.205 mm^3 in the older cohort, representing an 83% increase in mean tumor size over the 20-week observation period in untreated mice. In contrast, the mice treated with 50 mg/kg/day of imatinib showed a slower growth rate over this observation period with a 46% increase in mean tumor size between the younger and older cohorts (0.526 mm^3 at 4 months of age immediately after treatment discontinuation; 0.766 mm^3 at 9 months of age after 12 weeks of treatment and 20 weeks of observation). Additionally, the mice treated with 100 mg/kg/day of imatinib demonstrated only a 57% increase in mean tumor size (0.490 mm^3 at 4 months of age immediately after treatment discontinuation; 0.771 mm^3 at 9 months of age after 12 weeks of treatment and 20 weeks of observation). Independent of the dose of imatinib, treated mice had lower mean tumor size immediately after treatment and after 20 weeks of observation post-treatment than control mice.

DISCUSSION

Plexiform neurofibromas are complex tumors arising within the nerve plexus that are often apparent in infants and young children with NF1 and afflict up to 50% of people with NF1. The growth of pNF, driven by somatic inactivation of *NF1* [26], can lead to significant neurologic impairment, pain, disfigurement, and even mortality [27–29]. While growth rates can be unpredictable, progression is often most pronounced during childhood and complete surgical resection, the only established therapy, is rarely curative [30, 31]. Recent advances in the development of GEMMs that accurately recapitulate the growth kinetics and hallmark histopathological features of human pNF have led to insights regarding the cells of origin

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and critical role for the tumor microenvironment in the genesis and progression of these tumors [4, 32]. Moreover, GEMMs have enabled testing of experimental therapeutics [5, 33–35], several of which have delivered promising results in subsequent clinical trials [15, 19].

Previous genetic and bone marrow transplant experiments in GEMMs have established that tumorigenic Schwann cells secrete excessive quantities of kit-ligand (SCF) [36] and that kit/kit-ligand is required for tumor initiation [5]. We previously demonstrated in *Nf1*$^{flox/-}$*;Krox20-Cre(+)* mice that treatment with imatinib, a potent inhibitor of c-kit, in mice with established pNF leads to reduction in tumor number and size [5], which translated into a phase 2 clinical trial demonstrating the first successful treatment for reducing pNF volume [15]. A recent study by Liao *et al.* showed that while tumorous Schwann cells are the primary source of SCF that mediates mast cell chemotaxis in pNF, a marked reduction in mast cells only slightly influences tumor progression [22]. Thus, it is possible imatinib's efficacy may be due to additional mechanisms beyond inhibition of c-kit. Alternatively, there are subtle differences between the Plp-Cre model used by Liao and colleagues that disrupts the c-kit ligand versus earlier adoptive transfer experiments that disrupt the c-kit receptor in all hematopoietic lineages [5] and may impact both mast cells and other c-kit positive cells as well.

While other effective targeted therapies, including selumetinib [19] have emerged, pNF regrowth following discontinuation of therapy and during drug holidays remains a challenge and complete responses to therapy are not observed. Furthermore, in preclinical models, preventative use of MEK inhibition has not altered the initial development of pNF [23]. The preclinical studies outlined here evaluated the pharmacologic potential of imatinib to fundamentally block tumor initiation and progression early in life.

By administering imatinib to *Nf1*$^{flox/flox}$*;PostnCre(+)* mice starting at 1 month of age, prior to known pNF development, we were able to determine that 12 weeks of treatment significantly reduced tumor initiation, even at 10% of the MTD of imatinib at 10 mg/kg/day. The impact of imatinib on tumor growth following therapy, however, is unclear and patient responses have been variable (personal communication, Dr. Kent Robertson). In the present GEMM study, we found that early imatinib therapy largely prevented pNF initiation and significantly impaired development of these tumors at later timepoints even after prolonged discontinuation of drug. It is thus possible that imatinib leads to ablation of the initiating Schwann cell progenitors or induces a durable state of senescence. Additionally, the growth rate and volume of tumors that do form, as determined by proximal nerve size, was significantly reduced in mice treated with early imatinib and then observed for a prolonged period of 20 weeks before euthanasia. These findings indicate a sustained effect of imatinib on tumor growth velocity even after cessation of treatment.

The observation that early imatinib administration can blunt the tumorigenic capcity of *Nf1*-deficient Schwann cell progenitors even after prolonged cessation of therapy is crucial in considering clinical treatment approaches. Specifically, these data support clinical evaluation of imatinib in infants and young children with NF1 associated pNF for prevention of tumor progression. This is an important consideration clinically since maximal pNF growth rates are seen in childhood and imatinib has been formulated for pediatric dosing down to 6

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months of age. As growth of pNF during adulthood often plateaus, successfully preventing volume increases in childhood could have sustained effects. The kinetics of rebound tumor growth after cessation of imatinib also warrants further exploration and it is critically important to collect and compare these clinical data amongst paitents undergoing treatment with other targeted therapies such as MEK inhibitors.

Imatinib is best recognized for its use in the treatment of chronic myelogenous leukemia [37] for which it received FDA approval in 2003. Its primary long-term side effects are growth retardation and impaired bone health [38, 39]. These potential adverse effects are a concern in the setting of germline *NF1*, but could be mediated via intermittent dosing strategies until there is evidence of growth stabilization of pNF. Such an approach could reduce drug toxicities while potentially eliminating the physical impairment, pain, and disfigurement so often associated with these tumors.

Furthermore, clonal adaptation driven by genetic heterogeneity and kinome reprogramming [40, 41] is a scenario that commonly occurs in response to targeted chemotherapies. A distinct advantage of imatinib is that it targets a pathway extrinsic to tumorigenic Schwann cell progenitors. By disrupting a critical step in tumor initiation, the kit/kit-ligand dependent recruitment of tumor inciting mast cells to the microenvironment, this approach may avoid classical selective pressures for clonal evolution and ultimately tumor resistance generated by inhibiting intrinsic tumor cell kinases. In the present study, tumors that did emerge in the genetically engineered mice treated preventatively with imatinib retained hallmark pNF-associated histopathological features and we did not observe evidence of MPNST transformation in the imatinib-treated or vehicle groups.

Overall, we found administration of low-dose imatinib to young $Nf1^{flox/flox}$;*PostnCre(+)* mice was sufficient to inhibit pNF genesis with durable response post-treatment, demonstrating promise for use of imatinib as a preventative agent for NF1 associated pNF. The next step is to identify the 25–50% of NF1 affected infants and young children who develop pNF [42, 43], where early pharmacologic interventions may have the largest benefit. Discovery of circulating biomarkers capable of detecting nascent, asymptomatic tumors during the earliest stages of initiation, otherwise unidentifiable by conventional imaging modalities, represents a potential step towards this goal. Further exploration of such approaches in the context of NF1 associated pNF is of critical importance to advancement of the field, thereby enabling future clinical trials aimed at early tumor prevention with imatinib or other targeted chemotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Key

GEMM	Genetically engineered mouse model
NF1	Neurofibromatosis type 1
MPNST	Malignant peripheral nerve sheath tumor
MTD	Maximum tolerated dose
pNF	Plexiform neurofibroma
SCF	Stem cell factor/kit-ligand

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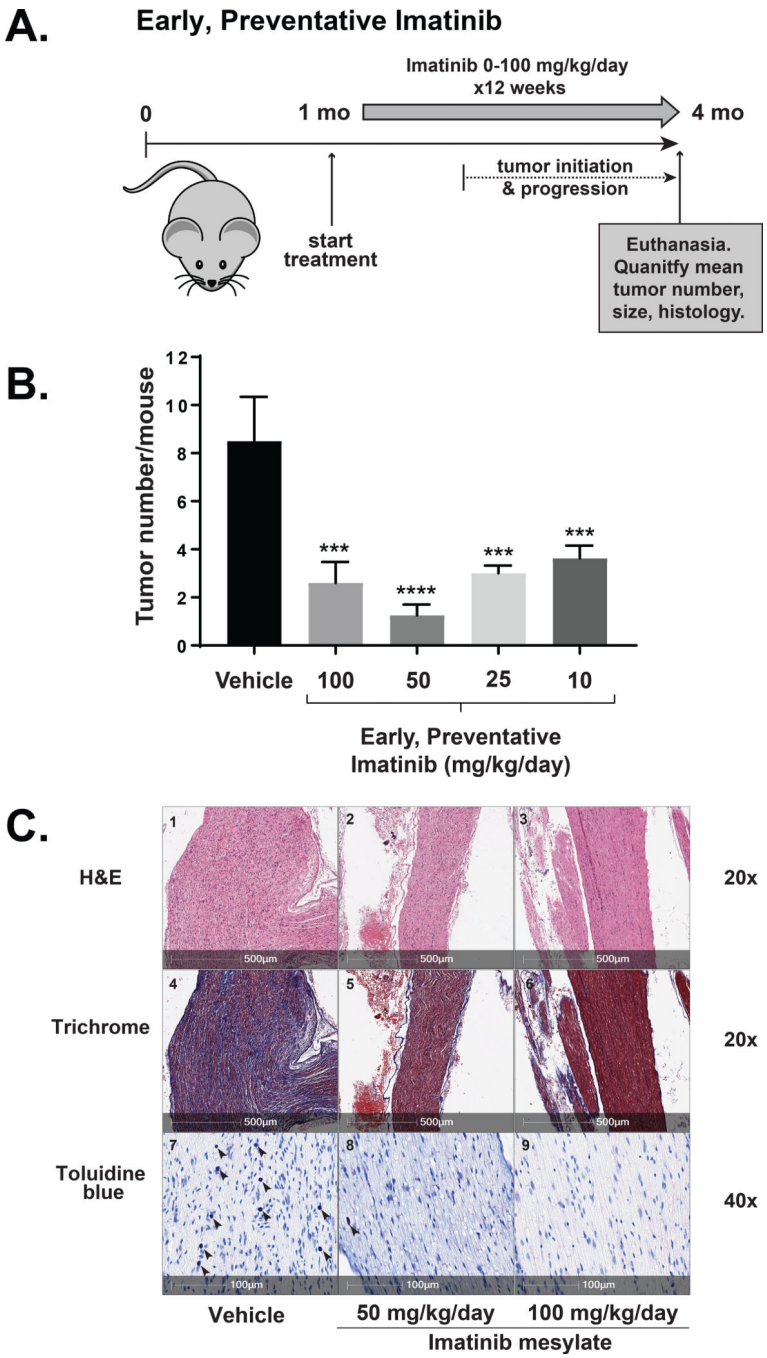

Figure 1. Early imatinib mesylate prevents plexiform neurofibroma initiation in young *Nf1*$^{flox/flox}$;*PostnCre(+)* mice.

(A) Schema depicting the experimental design. Cohorts of 1 month old *Nf1*$^{flox/flox}$;*PostnCre(+)* mice were treated with 0–100 mg/kg/day imatinib beginning prior to tumor onset (vehicle control, n=4; 10 mg/kg/day, n=8; 25 mg/kg/day, n=8; 50 mg/kg/day, n=8; 100 mg/kg/day, n=5). Treatment was continued for 12 weeks, and mice were then euthanized to assess tumor burden. **(B)** Decrease in mean number of plexiform neurofibromas at all doses of imatinib used: 100 mg/kg/day and 10 mg/kg/day

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(***p<0.001), 50 mg/kg/day and 25 mg/kg/day (****p<0.0001) as compared to vehicle treated controls. **(C)** Photomicrographs of hematoxylin and eosin stained sections demonstrating disrupted nerve architecture and increased cellularity in vehicle treated controls as compared to animals treated with imatinib 50–100 mg/kg/day (Panels 1–3). Masson's trichrome stained sections show abundant fibroblast collagen deposition in vehicle treated nerve sections which is absent in mice undergoing preventative imatinib treatment (Panels 4–6). Infiltrating mast cells, marked with black arrows on representative toluidine blue stained sections, are nearly absent in mice treated preventatively with imatinib (Panels 7–9).

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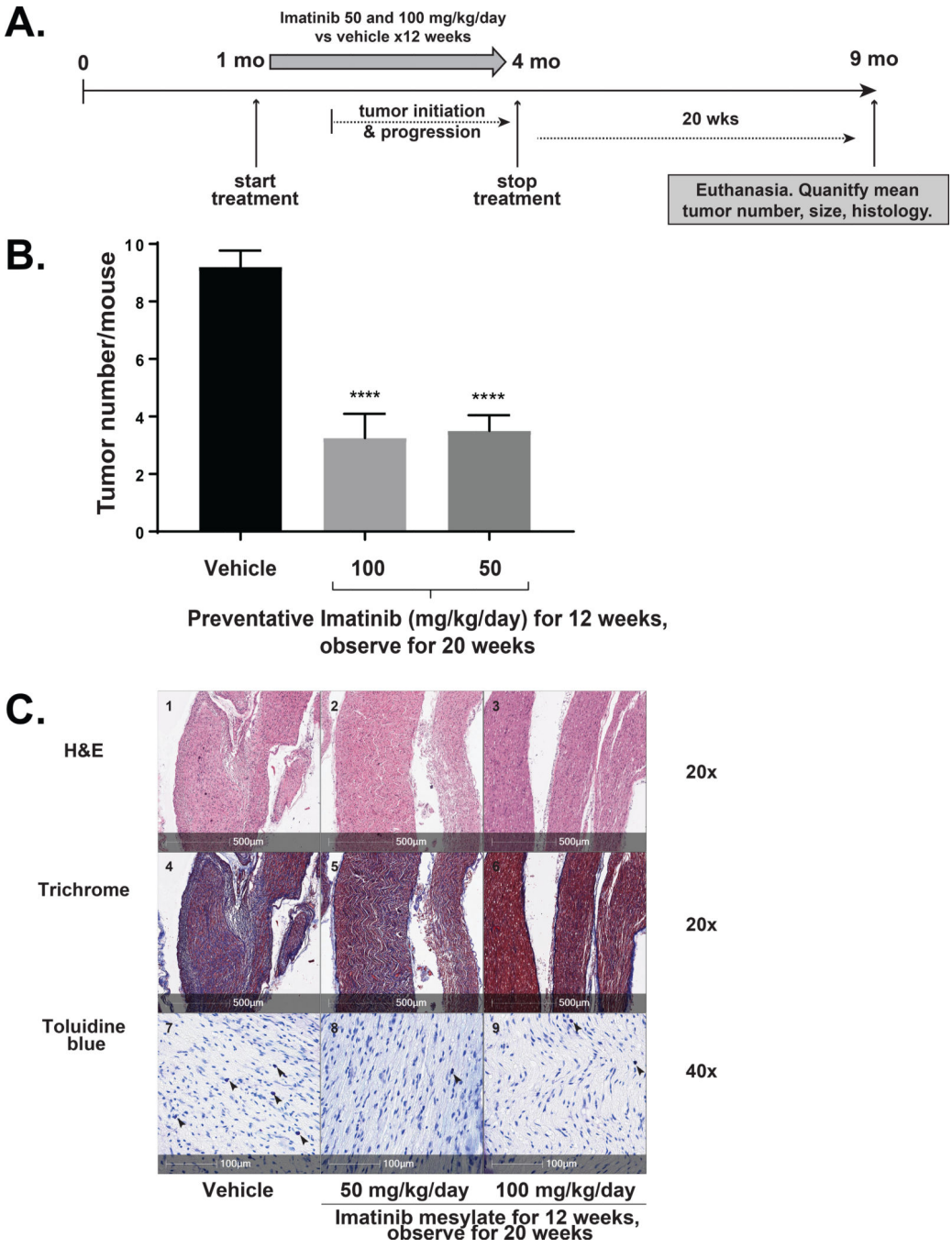

Figure 2. Sustained plexiform neurofibroma growth inhibition after early imatinib mesylate therapy followed by prolonged treatment cessation.

(A) Schema depicting the experimental design. Cohorts of 1 month old *Nf1*$^{flox/flox}$;*PostnCre(+)* mice were treated preventatively with 50 mg/kg/day or 100 mg/kg/day of imatinib for 12 weeks. Following discontinuation of therapy, mice were monitored for an additional 20 weeks prior to euthanasia to assess for tumor reemergence (vehicle control, n=10; 50 mg/kg/day, n=12; 100 mg/kg/day, n=12). **(B)** Significantly reduced mean plexiform neurofibroma number at 20 weeks after treatment discontinuation

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at both 50 and 100 mg/kg/day dosing levels (****p<0.0001). **(C)** Photomicrographs of hematoxylin and eosin stained sections demonstrating disrupted nerve architecture and increased cellularity in vehicle treated controls as compared to animals treated with imatinib 50–100 mg/kg/day and then observed for 20 weeks off treatment (Panels 1–3). Masson's trichrome stained sections show abundant fibroblast collagen deposition in vehicle treated nerve sections which is absent in mice undergoing imatinib treatment followed by observation (Panels 4–6). Infiltrating mast cells, marked with black arrows on representative toluidine blue stained sections, are notably diminished in mice treated with imatinib and then observed (Panels 7–9).

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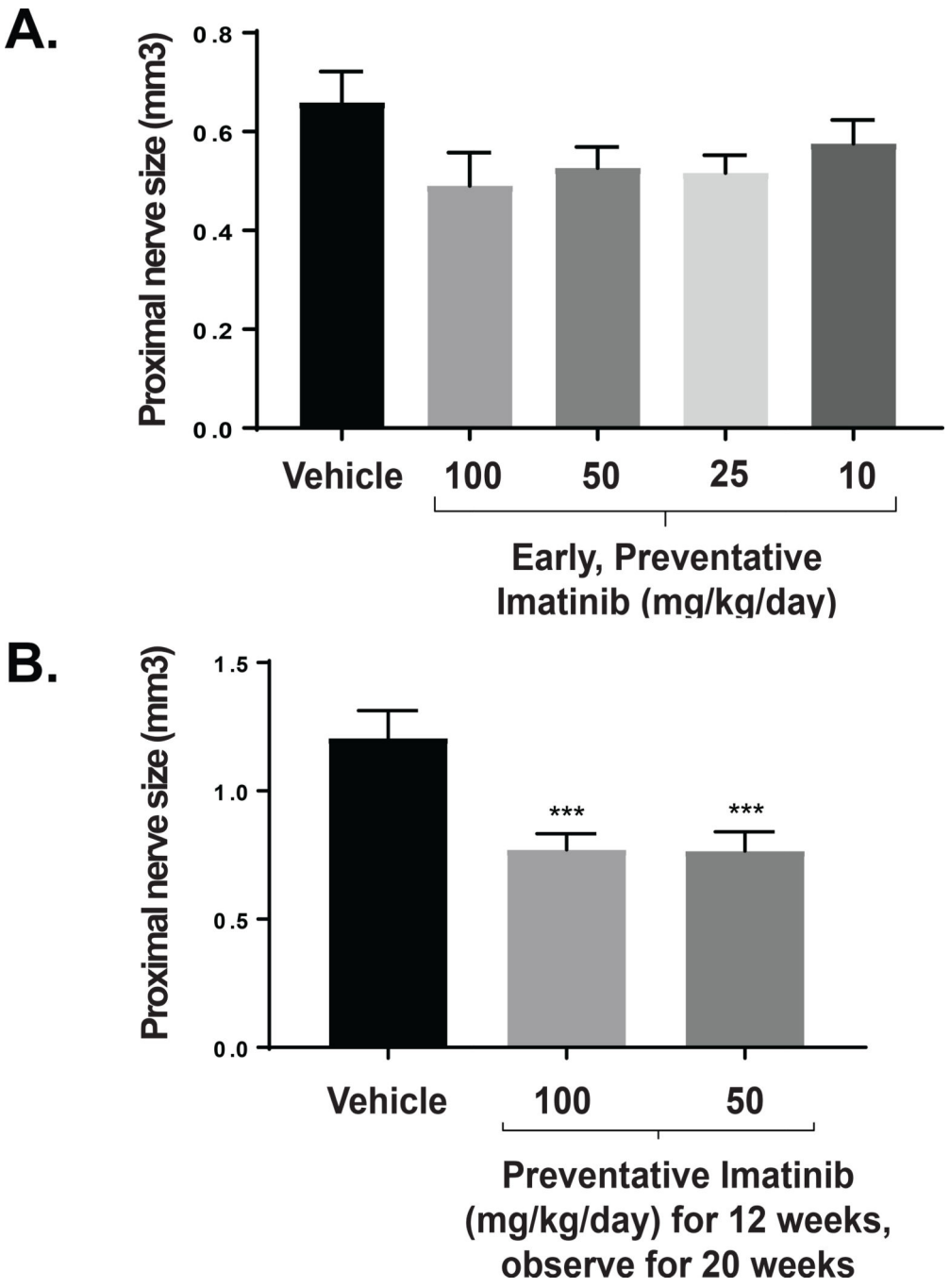

Figure 3. Early imatinib mesylate treatment reduces residual tumor volume in *Nf1*$^{flox/flox}$;*PostnCre(+)* mice treated preventatively and then observed off treatment.

(A) There was no significant difference in proximal nerve size, an estimation for tumor volume, in 4 month old *Nf1*$^{flox/flox}$;*PostnCre(+)* mice following 12 weeks of imatinib versus vehicle control (vehicle control, n=4; 10 mg/kg/day, n=8; 25 mg/kg/day, n=8; 50 mg/kg/day, n=8; 100 mg/kg/day, n=5). **(B)** In 9 month old *Nf1*$^{flox/flox}$;*PostnCre(+)* mice initially treated with 50 or 100 mg/kg/day of imatinib for 12 weeks starting at 1 month of age, and then observed off treatment for 20 weeks, there was a significant reduction in proximal nerve size

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in comparison to vehicle control (vehicle control, n=10; 50 mg/kg/day, n=12; 100 mg/kg/day, n=12) (***p<0.001).