Neurofibromatosis-1 Heterozygosity Increases Microglia in a Spatially and Temporally Restricted Pattern Relevant to Mouse Optic Glioma Formation and Growth

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Abstract

Whereas carcinogenesis requires the acquisition of driver mutations in progenitor cells, tumor growth and progression are heavily influenced by the local microenvironment. Previous studies from our laboratory have used Neurofibromatosis-1 (NF1) genetically engineered mice to characterize the role of stromal cells and signals to optic glioma formation and growth. Previously, we have shown that Nf1+/− microglia in the tumor microenvironment are critical cellular determinants of optic glioma proliferation. To define the role of microglia in tumor formation and maintenance further, we used CD11b-TK mice, in which resident brain microglia (CD11b+, CD68+, Iba1+, CD45low cells) can be ablated at specific times after ganciclovir administration. Ganciclovir-mediated microglia reduction reduced Nf1 optic glioma proliferation during both tumor maintenance and tumor development. We identified the developmental window during which microglia are increased in the Nf1+/− optic nerve and demonstrated that this accumulation reflected delayed microglia dispersion. The increase in microglia in the Nf1+/− optic nerve was associated with reduced expression of the chemokine receptor, CX3CR1, such that reduced Cx3cr1 expression in Cx3cr1-GFP heterozygous knockout mice led to a similar increase in optic nerve microglia. These results establish a critical role for microglia in the development and maintenance of Nf1 optic glioma.

Key Words: Astrocytoma, Chemokine, Fractalkine receptor, Microenvironment, Optic glioma, Stroma.

INTRODUCTION

The concept that the tumor microenvironment plays instructive roles in oncogenesis and cancer growth has gained considerable traction during the past 10 years (1). In other cancers, stroma-tumor interactions involve immune system cells (leukocytes, mast cells, and macrophages) and fibroblasts, which secrete extracellular proteins, cytokines, and growth factors (2–5). Unlike other cancers, fibroblasts and mast cells are not relevant stromal cell types in brain tumors (gliomas), suggesting that other cell types may contribute to microenvironmental influences on central nervous system (CNS) tumorigenesis and growth. Moreover, the stromal signals that influence glioma formation and growth are likely distinct from those implicated in non-CNS cancers and may be regulated in spatial and temporal patterns during postnatal brain development.

Previous studies from our laboratory have used Nf1 genetically engineered mouse (GEM) strains to define the role of the tumor microenvironment in glioma formation and growth. Neurofibromatosis-1 is a common tumor predisposition syndrome in which children are prone to the development of low-grade glial cell neoplasms (gliomas) along the optic pathway (optic pathway gliomas). These low-grade gliomas are classified as World Health Organization (WHO) grade 1 pilocytic astrocytomas and are typically found in the optic nerve and chiasm of children younger than 7 years (6). Children with NF1 are born with 1 nonfunctional copy of the Nf1 gene in all cells of their body (Nf1 heterozygosity; Nf1+/−) but develop gliomas only upon loss of the remaining functional allele in glial progenitor cells. To model this genetic condition in mice, we previously generated mice with Nf1 inactivation in glial progenitors; however, none of these mice developed gliomas (7). In contrast, glioma formation was only observed in Nf1+/− mice with glial cell Nf1 inactivation, thus establishing an absolute requirement for Nf1 heterozygosity in glioma formation (8). The observation that Nf1 loss in glial progenitors is necessary, but not sufficient, for tumorigenesis supported the hypothesis that nonneoplastic Nf1+/− cells provide a permissive environment required for glioma formation. By leveraging Nf1 GEM strains with highly penetrant tumor phenotypes, we showed that microglia represented one of the critical cell types present in the tumor microenvironment of Nf1 optic glioma mice (9).

Microglia have long been recognized as a major cellular constituent of the glioma microenvironment (10–13).
Rio-Hortega and Asua (14) first identified microglia in brain tumors in 1921; however, their role in glioma formation and continued growth (maintenance) remains unclear. In this regard, microglia may have antitumor activity (15, 16) or promote glioma growth by producing cytokines, such as interleukins 1 and 10, basic fibroblast growth factor, and transforming growth factor β (17–19). To define the role of microglia in glioma growth, we previously used 2 independent approaches to microglia inactivation in Nf1 GEM optic gliomas. First, using minocycline to inactivate microglia in 10- to 12-week-old Nf1 optic glioma mice, we found that reduced microglia activation, as assessed by morphological change, reduced tumor proliferation (20). Second, we identified that optic glioma mice, we found that reduced microglia activation, but the inhibitors used in these studies did not directly target microglia, but not Nf1-deficient astroglial cells, exhibited hyperactivation of c-Jun-NH2 kinase (JNK) such that treatment of Nf1 optic glioma mice with a relatively nonselective pharmacologic JNK inhibitor attenuated tumor proliferation (21). These proof-of-principle experiments demonstrated that Nf1+/− microglia inactivation decreased Nf1 optic glioma proliferation in vivo, but the inhibitors used in these studies did not directly target microglia and may have had additional effects. Moreover, these studies did not address the importance of microglia during Nf1 optic glioma development.

In the current study, we use a novel CD11b-TK transgenic mouse strain to ablate microglia genetically and demonstrate that microglia are important stromal cell types during gliomagenesis (tumor development) and for glioma maintenance. In addition, we define a spatially and temporally restricted window in which increased numbers of microglia are present in the Nf1+/− optic nerve. We further demonstrate that this increase in microglia likely reflects impaired microglial dispersal during postnatal optic nerve development as a result of reduced CX3CR1 expression. Collectively, these findings establish a critical role for microglia in promoting tumor proliferation throughout gliomagenesis.

**MATERIALS AND METHODS**

**Mouse and Human Specimens**

Mice were used in accordance to established Animal Studies Protocols at the Washington University School of Medicine. Nf1flox−/−; GFAP-Cre (Nf1+/−GFAP; KO), Nf1flox−/−; GFAP-Cre; CD11b-TK (Nf1+/−GFAP; CO-TK), CD11b-TK (22), Cx3cr1+/−; GFAP (23), Nf1−/−; Cx3cr1+/−;GFAP, wild-type (WT), and Nf1−/− mice were all maintained on a C57BL/6 background. The Nf1−/−;GFAP; CO-TK optic gliomas lack the Rosenthal fibers and eosinophilic granular bodies that are characteristic of human pilocytic astrocytomas but otherwise closely resemble pediatric low-grade gliomas based on the presence of discrete optic nerve/chiasm masses composed of hyperproliferating astrocytes with large hyperchromatic nuclei, low proliferative indices, new blood vessel formation, microglia infiltration, Olig2-immunoreactive cells, and contrast enhancement on small-animal magnetic resonance imaging (8); there is no evidence of endothelial hyperplasia or necrosis.

Human tumor specimens were used in accordance to established guidelines under an active and approved human studies protocol. Human glioma specimens were studied using 2 individual tissue microarrays developed at the Mayo Clinic Foundation and Washington University School of Medicine, as previously reported (24, 25). Nonneoplastic human brain samples from the neocortex were obtained from autopsy materials acquired under an approved human studies protocol at the Washington University School of Medicine.

**Immunohistochemistry**

Mice were perfused transcardially with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4). Optic nerves and optic chiasm were dissected and postfixed in 4% paraformaldehyde overnight at 4°C. All specimens were then processed for paraffin embedding and sectioning at the Ophthalmology Histology Core or the Histology and Microscopy Core at Washington University School of Medicine. Sections were deparaffinized in xylene and subjected to antigen retrieval. After the washing and blocking steps, sections were incubated overnight with rabbit anti-Iba1 (1:3000; Wako, Richmond, VA), mouse anti-Ki67 (1:500; BD Biosciences, San Jose, CA), or rat anti-CD34 (1:50; BD Biosciences) followed by incubation with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA) at room temperature (RT) for 1 hour. Immunoreactivity was visualized with the Vectastain ABC system and 3,3′-diaminobenzidine, Vector NovaRED, or Vector SG (Vector Laboratories).

All sections were photographed using a digital camera attached to a Nikon Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY). For each optic nerve counted, 3 photographs were taken at a magnification of 10× for at least 4 mice/group, including the optic chiasm and the right or left optic nerve just anterior to the chiasm (Figure, Supplemental Digital Content 1, part A, http://links.lww.com/NEN/A199). For brainstem and neocortex regions, 4 photographs were taken at a magnification of 10× for at least 4 mice/group (Figure, Supplemental Digital Content 1, parts B, C, http://links.lww.com/NEN/A199). The total number of Iba1+ cells was quantitated in the mouse prechiasmatic optic nerves, chiasm, brainstem, and neocortex, as previously described, with the investigator blinded to the genotype and treatment (26). All Iba1+ cells were counted regardless of morphology (amoeboid vs ramified). In the human tumors, the number of Iba1+ cells was calculated as a percentage of the total number of nucleated cells.

**Immunofluorescence**

After transcardial perfusion with Ringer’s solution (147 mmol/L NaCl, 4 mmol/L KCl, 2.2 mmol/L CaCl2·2H2O, with 2.5 USP U/ml heparin sodium injection, 0.02 mg/mL lidocaine HCl), WT mouse optic nerves and chiasm were dissected and postfixed in 4% paraformaldehyde overnight at 4°C. Tissues were cryoprotected in 30% sucrose in 0.1 mol/L sodium phosphate buffer (pH 7.4), embedded in OCT medium (Tissue-Tek, Miles, Inc., Elk hart, IN), cut into 10-µm sections in a cryostat, collected on Superfrost Plus slides (Fisher Scientific, Waltham, MA), and stored at −20°C. Slides were washed; permeabilized in 0.2% Triton X-100 in phosphate buffer; blocked; incubated overnight at 4°C with rabbit anti-Iba1 (1:2000), rat anti-CD11b (M1/70, 1:10; BD Biosciences), or mouse anti-Ki67 (1:500; BD Biosciences);
and followed by incubation with fluorescent secondary antibodies (1:200 goat anti-rat A488, goat anti-rabbit A568, or goat antimouse A488; Invitrogen, Carlsbad, CA) for 1 hour at RT. In all cases, no specific immunostaining was observed in the negative control samples. Images were acquired using an Olympus multichannel confocal microscope (Olympus America Inc., Center Valley, PA) in the Washington University Bakewell Neuroimaging Core Facility, and Image J image analysis software (http://rsbweb.nih.gov/ij/; Wayne Rasband, National Institute of Mental Health, Bethesda, MD) was used to obtain a single collapsed image from an optical stack.

Ganciclovir Treatment

Ganciclovir (GCV; Sigma-Aldrich, St Louis, MO) was dissolved in phosphate-buffered saline (PBS) and administered at a dosage of 50 mg/kg. For the experiments focused on determining the acute effects of microglia ablation on optic glioma proliferation, Nf1<sup>j−/−;GFP<sup>CKO</sup>-TK</sup> mice were divided into two groups: one group received daily intraperitoneal GCV injections for 2 weeks starting at 3 weeks or 3 months, whereas the other group received daily intraperitoneal injections of vehicle (PBS) starting at 3 weeks or 3 months.

Flow Cytometry

Microglia from mice were collected using previously described procedures with minor modifications (27). Briefly, for each experiment, optic nerves and chiasm harvested from 7 to 10 mice, transcardially perfused with Ringer’s solution, were homogenized through a 70-μm nylon filter (Fisher Scientific), enzymatically digested in Hanks’ buffered salt solution without calcium and magnesium containing 0.05% collagenase I (Sigma-Aldrich), 0.1 μg/mL Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK; Sigma-Aldrich), 0.01 mg/mL bovine deoxyribonuclease I (DNase I; Sigma-Aldrich), 10 mmol/L HEPES buffer (pH 7.4) for 1 hour at RT, and filtered through a 40-μm nylon filter (Fisher Scientific). Brainstem specimens were similarly processed to obtain cells and filtered through a 40-μm nylon filter (Fisher Scientific). Brainstem specimens were similarly processed to obtain cells.

Quantitative Reverse Transcription–Polymerase Chain Reaction

Real-time polymerase chain reaction (quantitative PCR, qPCR) was performed using SYBR Green detection according to the manufacturer’s instructions. Total RNA was extracted from Nf1<sup>j−/−</sup> and WT optic nerve using TRIZol. Complementary DNA was synthesized from total RNA samples using the Ominiscript kit (Qiagen, Alameda, CA). Amplification was performed in 96-well plates in a real-time sequence detection system instrument (CFX96 Real-Time System; Bio-Rad, Hercules, CA). Primer sequences for Cx3cr1 were 5′-AGGGCTTGT TATTTGGGCCCAGAT-3′ (forward) and 5′AGCGAGACCCA CAAACGATT-3′ (reverse). Primer sequences for Cx3crl were 5′-AAATGGGTCCAAGACGCCATGA-3′ (forward) and 5′-ACATTGTCCACCCGCTTCCTCAAA-3′ (reverse). Bio-Rad CFX Manager software was used to convert the fluorescent data into cycle threshold (CT) measurements. The ΔΔCT method was used to calculate changes in fold expression relative to WT optic nerve using β-actin as an internal control.

Statistical Analyses

Student t tests, along with Grubbs test to determine outliers, were performed for all statistical analyses. Mean and SEM were used for all graphs. For all experiments involving cell quantitation, the investigators were blinded to mouse genotype and treatment.

RESULTS

Increased Microglia in NF1-Associated Pilocytic Astrocytomas

Several previous studies have reported increased numbers of microglia in human gliomas, constituting as many as a third of all cells in glioma biopsies (10–13). We also found a 2-fold increase in the percentage of Iba1-positive cells (microglia) in high-grade human astrocytomas (Figs. 1A, B). Previous studies have suggested that Iba1 is a more selective marker of brain microglia in both human (29) and rodent tissues (30). By contrast, anti-CD68 antibodies label resident microglia and magnetic cell sorting using CD11b microbeads after myelin removal (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to extract the CD11b+ microglia population. After Fc microglia sample from the 37% to 70% interface. Alternately, 70% allowed for collection of an enriched macrophage and neutrophils (CD68+, CD11b+, CD45 high) (33, 34), dendritic cells (CD68+, CD11b+, CD45<sup>high</sup>) (35, 36), and bone marrow–derived monocytes (CD68<sup>+</sup>, CD11b<sup>+</sup>, CD45<sup>high</sup>) (32, 39). Similar percentages of microglia were generally found in WHO grade 1 low-grade gliomas as in the high-grade tumors, but NF1-associated pilocytic astrocytomas (NF1-PA) had higher percentages of microglia than their sporadic counterparts (SP-PA) (p = 0.0008) (Figs. 1A, C).

Because previous reports used CD68 as a marker for microglia in brain tumors (40, 41), we analyzed numbers of CD68<sup>−</sup> cells in the PA tumors. There was a trend toward...
increased numbers of CD68+ cells in NF1-PA tumors versus both their sporadic counterparts and nonneoplastic brain (NB) tissue, the results did not reach statistical significance (Figure, Supplemental Digital Content 2, parts A, B, http://links.lww.com/NEN/A200). We suspect the differences between the results obtained using Iba1 and CD68 immunostaining reflects the labeling of blood-derived macrophages and neutrophils by the anti-CD68 but not the Iba1 antibody. We also observed stronger immunoreactivity with the Iba1 antibody in the formalin-fixed, paraffin-embedded specimens. Moreover, in studies of CD68 and Iba1 colabeling in areas of brain ischemia, numerous CD68+ cells lacked Iba1 colabeling and, therefore, likely represented bone marrow-derived macrophages, leukocytes, neutrophils, or dendritic cells (35, 39) (Figure, Supplemental Digital Content 2, part C, http://links.lww.com/NEN/A200). The finding of greater numbers of Iba1+ cells in NF1-PA than in SP-PA raised suggested that these stromal cells play a particularly important role in NF1-associated gliomas.

FIGURE 1. Increased microglia in neurofibromatosis type 1 (NF1)–associated pilocytic astrocytomas. (A–C) Iba1 immunohistochemistry demonstrates increased percentages of microglia in anaplastic astrocytomas (AA) (p = 0.0236) and glioblastoma multiforme (GBM) tumors (p = 0.0377) versus nonneoplastic brain (NB). (C) Sporadic pilocytic astrocytomas (SP-PA) have a greater percentage of Iba1+ cells compared with NB (p < 0.0001) (A, C), but the greatest percentage of microglia is seen in NF1-associated pilocytic astrocytomas (NF1-PA) (p < 0.0001 versus NB) (p = 0.0008 versus SP-PA).

Genetic Ablation of Microglia in Established Tumors Decreases Nf1 Mouse Optic Glioma Proliferation

Our previous study on microglia in human pilocytic astrocytomas suggested that the number of microglia in the tumors positively correlated with glioma proliferation rate (42). We also previously showed that minocycline- or JNK inhibitor–mediated microglia inactivation decreased optic glioma tumor cell proliferation in vivo (20). However, these pharmacologic approaches are limited by potential off-target effects affecting other cell types in the tumor microenvironment and do not directly address the role of microglia in tumor proliferation. To establish a more definitive role for microglia in regulating Nf1 optic glioma proliferation in Nf1+/−GFAPCKO mice at 3 months with radiographically and histologically obvious tumors (glioma maintenance), we used a novel transgenic mouse in which CD11b+ cells express the thymidine kinase (TK) gene (22). On the administration of GCV, CD11b+ cells are killed.
To characterize the CD11b+ population in the mouse optic nerve and to determine whether the ablated CD11b+ cells were primarily microglia, we performed flow cytometry and immunohistochemistry studies using several established monocye markers. First, we found that CD11b and CD68 label the same population of cells in the optic nerves of 6-week-old WT mice (Fig. 2A). Most of this population expressed low levels of CD45 characteristic of resident microglia (R1 = 95% of the CD11b+ cell population) rather than high levels of CD45 indicative of bone marrow–derived macrophages (Fig. 2B). Iba1 co labeled these CD11b+ cells, as determined by flow cytometry (Fig. 2C) and Iba1/CD11b double labeling in optic nerve sections (Fig 2D). In both cases, more than 99% of the cells analyzed were positive for both CD11b and Iba1. Iba1+ cells also express low levels of CD45 (Fig. 2E; R1 = 95% of Iba1+ cells), consistent with their classification as resident microglia. Moreover, using CD11b magnetic bead capture of microglia from optic gliomas of 3-month-old Nf1+/-GFAP CKO mice, most of the CD11b+ population isolated had low CD45 expression (Fig. 2F), thereby establishing that GCV/TK–mediated ablation of CD11b+ cells in Nf1 mouse optic gliomas mainly targets the resident microglia population.

On the basis of these findings, we intercrossed CD11b-TK mice with our Nf1+/-GFAP CKO mice to generate Nf1+/-GFAP CKO-TK mice and injected GCV intraperitoneally daily for 2 weeks into 3-month-old Nf1+/-GFAP CKO-TK mice. For controls, vehicle (PBS) was injected into 3-month-old Nf1+/-GFAP CKO-TK mice. At the completion of the 2-week treatment, the optic nerves were removed for analysis. After GCV administration, there was a 49% reduction in the numbers of Iba1+ microglia in the optic nerves of the 3-month-old GCV-treated versus vehicle-treated control mice (Fig. 3A). No differences in numbers of Iba1+ cells or Ki67-positive cells between PBS-treated Nf1+/-GFAP CKO-TK mice were found compared with GCV-treated Nf1+/-GFAP CKO mice lacking the CD11b-TK transgene (data not shown). Consistent with previous studies using minocycline (20), GCV-mediated microglia ablation reduced optic glioma proliferation (Ki67-positive staining), by 94% (Fig. 3B). This reduction in proliferation reflects reduced glial cell proliferation and not microglia proliferation, as demonstrated by a lack of double labeling for Ki67 and Iba1 (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A201) (20). In contrast to the effects of GCV treatment on microglia cell numbers and optic glioma proliferation, there was no significant change in the number of CD34+ cells within the optic nerves of GCV-treated Nf1+/-GFAP CKO-TK mice compared with vehicle-treated controls (Fig. 3C). Together with previous studies, these new findings establish a critical role for microglia in the maintenance of Nf1 mouse optic glioma proliferation.

Microglia Ablation During Optic Glioma Development Reduces Tumor Proliferation

One of the unique opportunities afforded by GEM tumor models is the ability to define the role of stromal cell types during tumor evolution. We hypothesized that microglia might also have important functions during optic glioma formation that may be distinct from their growth-promoting role in glioma maintenance. Previously, we showed that the earliest changes in optic nerve organization were observed between 3 and 6 weeks, as evidenced by increased numbers of microglia (20), increased glial cell proliferation (9, 26), and disruption of the relationship between optic nerve axons and their associated glia (43). To determine the impact of microglia inactivation/ablation during the early phase of optic glioma formation, we treated Nf1+/-GFAP CKO-TK mice with GCV or control vehicle (PBS) for 2 weeks beginning at the age of 3 weeks.

Similar to the GCV-treated 3-month-old Nf1+/-GFAP CKO-TK mice, there was a 43% decrease in the number of Iba1+ cells in the optic nerves of GCV-treated mice at 3 to 5 weeks versus controls (Fig. 4A). After GCV treatment, optic glioma proliferation was decreased by 63% (Fig. 4B), with no change in the number of CD34+ cells (Fig. 4C). These results demonstrate for the first time that microglia also facilitate glial cell proliferation during the early phases of optic glioma development.

Nf1+/- Optic Nerves Have Increased Numbers of Microglia at the Age of 6 Weeks But Not 3 Weeks or 3 Months

We next sought to determine whether Nf1 heterozygosity resulted in a temporally and spatially restricted increase in microglia numbers. For these studies, we performed Iba1 immunostaining in the optic nerves and brains of Nf1+/- and WT mice at 2 time points: before obvious glioma formation (3 weeks and 6 weeks) and a time point when radiographically and histologically evident gliomas are seen (3 months). At 3 weeks, Nf1+/- and WT mice had equivalent numbers of Iba1+ cells in the optic nerve, but at 6 weeks, microglia numbers in the WT optic nerve had decreased compared with the WT optic nerve at 3 weeks, whereas the number of microglia in the Nf1+/- optic nerve did not decline (Figs. 5A, B). This difference in microglia dispersion resulted in a 2-fold increase in the number of microglia in the optic nerves of Nf1+/- mice compared with their WT littermates. By 3 months, no differences in optic nerve microglia numbers were observed between Nf1+/- and WT mice (Figs. 5A, B). These results suggest the notion that there is developmental (temporal) regulation of microglia numbers in the optic nerve resulting from Nf1 heterozygosity, suggesting that Nf1+/- microglia have delayed or defective dispersal.

To determine whether this effect of Nf1 heterozygosity on microglia number was also spatially regulated, we counted microglia in the brainstem and neocortex of the Nf1+/- and WT mice. In striking contrast to the temporal effects of Nf1 heterozygosity in optic nerves, no differences in brainstem or neocortex microglia numbers were observed at 3 weeks, 6 weeks, or 3 months between Nf1+/- mice and their WT littermates (Figs. 5C, D). Thus, Nf1 heterozygosity results in both a temporal and spatial increase in microglia within the mouse optic nerve.

CX3CR1 Expression Is Reduced in the Optic Nerves of Nf1+/- Mice

One of the key chemokines that regulate microglia dispersal in the brain is fractalkine or CX3CL1 (44, 45).
CX3CL1 acts on its receptor, CX3CR1, to regulate microglia migration and function (46), and loss of CX3CR1 expression has been reported to lead to subretinal microglia cell accumulation (47). To determine whether altered CX3CL1/CX3CR1 expression could account for abnormal accumulation of microglia in the optic nerves of 6-week-old Nf1+/− mice, we first measured Cx3cl1 mRNA levels by qPCR in Nf1+/− and WT optic nerves at different ages. No differences in Cx3cl1 expression were observed in Nf1+/− optic nerves relative to their WT counterparts at any time point (Fig. 6A).

FIGURE 3. Microglia ablation at 3 months reduces optic glioma proliferation. (A, B) Treatment of Nf1+/−GFAP^KO-TK^ mice (n = 7) with ganciclovir (GCV) at 3 months reduces the number of Iba1+ cells by 49% (p = 0.0280) and results in a 94% decrease in the number of Ki67-positive cells in the optic nerve (p = 0.0118) versus Nf1+/−GFAP^KO-TK^ mice treated with vehicle (n = 7). (C) Ganciclovir treatment did not affect endothelial cell numbers, as assessed by CD34 immunohistochemistry (p = 0.2864).

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FIGURE 2. Microglia in the mouse optic nerve at 6 weeks and in mouse optic gliomas at 3 months are mainly resident microglia. (A) Flow cytometry demonstrates that most cells from 6-week-old wild-type (WT) optic nerves (10 pooled optic nerves) are doubly positive for both CD11b and CD68. (B) Most of these CD11b+ cells express low levels of CD45 (R1). (C) CD11b+ microglia in the 6-week-old optic nerve are also Iba1+. (D) Using double-labeling immunofluorescence, there are rare cells in the 6-week-old WT optic nerve that label with CD11b antibodies (green) alone (inset, arrowhead), whereas most (~99%) of the microglia colabel with both CD11b and Iba1 antibodies (inset, arrows) (n = 5). (E) Iba1+ cells express low levels of CD45 (R1). (F) CD11b magnetic cell capture of microglia from 3-month-old Nf1+/−GFAP^KO^ mouse optic gliomas (7 pooled optic nerves) followed by flow cytometry demonstrates that the CD11b+ cells are also almost exclusively CD45^low^ (R1).

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Next, we examined Cx3cr1 mRNA expression by qPCR using total RNA isolated from Nf1+/- and WT mice optic nerves at different ages. There was a trend increase toward increased Cx3cr1 mRNA expression at 3 weeks in Nf1+/- optic nerves (p = 0.0855), whereas there was a 2.5-fold decrease in Cx3cr1 mRNA in Nf1+/- compared with WT optic nerves at 6 weeks (Fig. 6B). By 3 months, Cx3cr1 mRNA expression in the Nf1+/- optic nerves was equivalent to that in the WT mice. Attempts to confirm these results at the protein level were unsuccessful owing to the lack of suitable CX3CR1 antibodies for immunohistochemistry.

To determine whether reduced CX3CR1 expression leads to increased microglia accumulation in the optic nerve as observed in Nf1+/- mice, we used Cx3cr1+/GFP mice in which 1 copy of the Cx3cr1 gene was replaced with a complementary DNA encoding green fluorescent protein (GFP) (23). At 6 weeks, there were 2-fold more Iba1+ cells in the optic nerves of Cx3cr1+/GFP mice compared with WT controls (Fig. 6C). Similarly, there was an increase in the number of Iba1+ cells in the optic nerves of Nf1+/-; Cx3cr1+/GFP mice compared with Nf1+/- controls at 6 weeks (p < 0.0001). Collectively, these results suggest that Nf1+/- microglia exhibit a delay in dispersal from the optic nerve related to reduced CX3CR1 expression, resulting in increased numbers of microglia at a critical time during optic glioma development.

**DISCUSSION**

Gliomagenesis requires the combination of susceptible preneoplastic cells coupled with spatially and temporally...
restricted signals emanating from the tumor microenvironment. This cellular collaboration may account for the unique pattern of glioma formation within the CNS in children with NF1. With few exceptions, gliomas are predominantly located along the optic pathway and less frequently in the brainstem in young children. On the basis of this unique pattern of gliomagenesis, we previously showed that optic nerve and brainstem, but not the neocortex, astrocytes increase their proliferation in response to \( Nf1 \) gene inactivation in vitro and in vivo (48). This region-specific susceptibility to the effects of neurofibromin loss provides a receptive preneoplastic cell type, which, in cooperation with specific signals from the local environment, facilitates gliomagenesis.

The requirement for key stromal signals is illustrated by studies of \( Nf1 \) GEM strains. In both peripheral and CNS tumors, \( Nf1 \) loss in Schwann cell or glial cell precursors, respectively, is not sufficient for tumor formation (5, 7). Neurofibromas or gliomas only form when \( Nf1 \) inactivation occurs in Schwann or glial cell precursors in \( Nf1^{+/\text{-}} \) mice (5, 8, 49).

FIGURE 5. Iba1+ cells are increased in \( Nf1^{+/\text{-}} \) optic nerve (ON) at 6 weeks but not at 3 weeks or 3 months. (A, B) Equivalent numbers of Iba1+ cells were found in the ON of \( Nf1^{+/\text{-}} \) (n = 5) (black bars) and wild-type (WT) (gray bars) mice (n = 5) at 3 weeks (p = 0.9712). By 6 weeks, there was a 2-fold increase in the number of Iba1+ microglia in the ONs of \( Nf1^{+/\text{-}} \) mice (n = 5) versus WT littermates (n = 5) (p = 0.0002). By 3 months, \( Nf1^{+/\text{-}} \) (n = 4) and WT (n = 4) mice had equivalent numbers of Iba1+ cells (p = 0.5155). (C, D) \( Nf1^{+/\text{-}} \) mice (n = 5) have similar numbers of Iba1+ microglia in the brainstem (BS) and cortex (CTX) versus WT (n = 5) at 3 weeks (BS, p = 0.7531) (CTX, p = 0.5248), 6 weeks (BS, p = 0.8260) (CTX, p = 0.0911), and 3 months (BS, p = 0.8138) (CTX, p = 0.6162).
important stromal cell types in neurofibroma and optic glioma development and maintenance, respectively. In the current study, we show that NF1-associated human pilocytic astrocytomas have increased numbers of microglia and use several Nf1 GEM strains to establish a pivotal role for resident microglia in both optic glioma development and maintenance.

First, we demonstrated that astrocytic tumors of all malignancy grades harbor increased percentages of Iba1+ microglia versus nonneoplastic brain. Previous studies have used CD68 or Iba1 to identify microglia and found correlations between the malignancy grade and CD68/Iba1 immunopositivity (51, 52), but others have reported differences in microglia morphology (12) or proliferation (40) in gliomas of varying histologic grade. We observed a statistically insignificant trend toward increased CD68+ cells in gliomas versus nonneoplastic brain. We suspect that the difference between CD68 and Iba1 as microglia markers reflects the fact that CD68 recognizes other monocyte-like cells in addition to microglia and may also stain nonmonocyte/macrophage lineage cells, especially under pathologic conditions (33, 35, 39). Indeed, we demonstrated that macrophages in an ischemic region in the human brain were strongly CD68+, whereas few cells in that pathological focus coincidentally labeled with Iba1. These data strongly suggest that Iba1 is a more selective marker for brain microglia (29, 30) but also highlight the need to identify additional specific markers for resident microglia.

We found that GCV-mediated reductions in microglia numbers were similar during tumor evolution (5–6 weeks) and in the established glioma (3 months). Using this method to attenuate microglia function, we provide definitive evidence for the role of microglia in glioma proliferation in established tumors and show for the first time their role in glioma proliferation during tumor formation. Although this method of microglia ablation should target only CNS microglia, several independent experiments confirmed the identity of the CD11b+ cells. Using flow cytometry, we found that the normal optic nerve macrophage/monocyte population is primarily composed of CD11b+ CD45low cells, consistent with the profile of resident brain microglia (34, 53). Moreover, these microglia were CD68+ (by flow cytometry) and Iba1+ (by immunofluorescence and flow cytometry). Similar to our previous studies in human NF1-associated optic gliomas (20), the CD11b+ population in Nf1+/−/GFAP−/− mice optic gliomas was CD45low resident brain microglia. Together, these results support the conclusion that genetic elimination of resident microglia reduces optic glioma proliferation both during tumor formation and tumor maintenance.

Because of the limited reagents currently available to characterize brain microglia, it is possible that there are different populations of microglia in the optic nerve at 5 to 6 weeks and 3 months. In an analogous fashion, macrophages

![Graph A](image1.png)

**Figure 6.** Reduced CX3CR1 expression in the Nf1+/− optic nerve (ON). (A) Real-time quantitative polymerase chain reaction (PCR) reveals no change in Cx3cl1 mRNA levels in ONs from Nf1+/− (n = 5) (black bars) and wild-type (WT) (n = 5) (gray bars) mice at 3 weeks (p = 0.1558), 6 weeks (p = 0.3478), or 3 months (p = 0.4788). (B) Quantitative PCR reveals a greater than 2-fold decrease in Cx3cr1 mRNA expression in the ONs of 6-week-old Nf1+/− mice (n = 5) versus WT controls (n = 5) (p = 0.0181). There is no difference in Cx3cr1 expression in the ONs from Nf1+/− versus WT mice at 3 weeks (p = 0.0855) or 3 months (p = 0.1413). (C) There is a 2-fold increase in numbers of Iba1+ microglia in ONs of Nf1+/− mice (n = 5) versus WT controls (n = 5) (p = 0.0042). Nf1+/− mice (n = 7) have greater numbers of microglia versus WT (p < 0.0001) and Nf1+/− mice (n = 5) (p < 0.0001).
have been proposed to have varying functions relevant to tumorigenesis as a function of tumor evolution (54, 55). Early during tumor development, macrophages are active participants in eliminating tumor cells, but as the stroma and cancer cells coevolve an “equilibrium” phase emerges in which the neoplastic cells acquire resistance to immune editing and elimination (56, 57). Finally, these “adapted” tumor cells expand and escape from immunologic pruning and create a new condition where macrophages may facilitate tumor growth. It is not clear whether these phases exist for microglia-glioma cell interactions, but if so, it will be important to identify specific subpopulations of microglia with unique properties germane to a given period of glioma development and maintenance.

We also showed that Nf1 heterozygosity creates spatially and temporally restricted differences in microglia abundance relevant to optic glioma evolution. The fact that the numbers of Iba1+ microglia increase in Nf1+/− mice in the optic nerve during a defined window of development suggests that this specific microglial population might be uniquely susceptible to the effects of reduced Nf1 expression during this period of optic nerve maturation. We hypothesize that the accumulation of Nf1+/− microglia in the optic nerve at 5 to 6 weeks reflects a defect in microglia homing in response to chemokines that instruct microglia dispersal. It is unlikely that Nf1+/− optic nerve microglia have impaired migratory abilities as we have previously shown that Nf1+/− microglia have increased motility in Boyden chamber assays in vitro (20, 21). Rather, we propose that Nf1+/− microglia are defective in directed migration.

One of the major determinants controlling microglia homing is CX3CR1 (58), which was reduced in the Nf1+/− optic nerve. Here, we showed that reduced Cx3cr1 expression in CX3CR1-GFP heterozygous knockout mice resulted in a similar increase in optic nerve microglia at 6 weeks. These results are consistent with previous reports demonstrating that Cx3cr1-deficient mice exhibit microglia accumulation in the retina (47, 59, 60). Together, these findings suggest a model in which microglia accumulation reflects the impact of Nf1 heterozygosity on the developmental pattern of microglia migration in the optic nerve, thus delaying microglia dispersal and resulting in the presence of microglia and microglia-produced signals during a time when Nf1+/− glial progenitors are most susceptible to expansion by stromal elements.

Previous studies have demonstrated the presence of CD11b+ Iba1+, CX3CR1+ microglia in human gliomas, leading to the hypothesis that this cell population might be important for glioma growth (61). Moreover, polymorphisms in the human CX3CR1 gene are linked to increased survival of patients with high-grade glioma, and patients with the common “improved survival” V249I polymorphism also had reduced numbers of tumor-associated microglia (62). These findings raise the intriguing possibility that neurofibromin regulation of CX3CR1 expression might control microglia function in a specific fashion to modulate neoplastic glial cell growth in both the evolving and established optic glioma. Collectively, our new observations suggest a mechanistic model of stroma-tumor cell interaction relevant to optic gliomagenesis and maintenance that envisions Nf1 heterozygosity as an essential event that alters microglia abundance during optic nerve development. Future studies aimed at defining the function of microglia during gliomagenesis and glioma maintenance will be required to begin to develop treatments that target the relevant microenvironmental cell types in this common pediatric brain tumor.

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REFERENCES

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