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Authors
Solga, Anne C
Pong, Winnie W
Kim, Keun-Young
et al.

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RNA Sequencing of Tumor-Associated Microglia Reveals Ccl5 as a Stromal Chemokine Critical for Neurofibromatosis-1 Glioma Growth

Anne C. Solga*, Winnie W. Pong*, Keun-Young Kim†, Patrick J. Cimino‡, Joseph A. Toonen*, Jason Walker§, Todd Wylie§, Vincent Magrini§, Malachi Griffith§, Obi L. Griffith§, Amy Ly§, Mark H. Ellisman†, Elaine R. Mardis§ and David H. Gutmann*

*Department of Neurology, Washington University School of Medicine, St. Louis, MO; †National Center for Microscopy and Imaging Research, University of California, San Diego, CA; ‡Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO; §The Genome Institute, Washington University School of Medicine, St. Louis, MO

Abstract
Solid cancers develop within a supportive microenvironment that promotes tumor formation and growth through the elaboration of mitogens and chemokines. Within these tumors, monocytes (macrophages and microglia) represent rich sources of these stromal factors. Leveraging a genetically engineered mouse model of neurofibromatosis type 1 (NF1) low-grade brain tumor (optic glioma), we have previously demonstrated that microglia are essential for glioma formation and maintenance. To identify potential tumor-associated microglial factors that support glioma growth (gliomagens), we initiated a comprehensive large-scale discovery effort using optimized RNA-sequencing methods focused specifically on glioma-associated microglia. Candidate microglial gliomagens were prioritized to identify potential secreted or membrane-bound proteins, which were next validated by quantitative real-time polymerase chain reaction as well as by RNA fluorescence in situ hybridization following minocycline-mediated microglial inactivation in vivo. Using these selection criteria, chemokine (C-C motif) ligand 5 (Ccl5) was identified as a chemokine highly expressed in genetically engineered Nf1 mouse optic gliomas relative to nonneoplastic optic nerves. As a candidate gliomagen, recombinant Ccl5 increased Nf1-deficient optic nerve astrocyte growth in vitro. Importantly, consistent with its critical role in maintaining tumor growth, treatment with Ccl5 neutralizing antibodies reduced Nf1 mouse optic glioma growth and improved retinal dysfunction in vivo. Collectively, these findings establish Ccl5 as an important microglial growth factor for low-grade glioma maintenance relevant to the development of future stroma-targeted brain tumor therapies.

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Introduction
Studies in various experimental model systems have demonstrated that cancers develop within complex tissue environments that dramatically influence tumor cell growth, transformation, and metastasis. Within the microenvironment of most solid tumors are a variety of nonneoplastic cell types, including fibroblasts, immune

Address all correspondence to: David H. Gutmann, MD, PhD, Department of Neurology, Box 8111, 660 South Euclid Ave, Washington University School of Medicine, St. Louis MO 63110.
E-mail: gutmannd@neuro.wustl.edu

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system cells, and endothelial cells. Each of these stromal cell types has the capacity to produce growth/survival factors, chemokines, extracellular matrix, and angiogenic molecules that can change the local milieu in which neoplastic cells grow and infiltrate. Although the importance of the cancer microenvironment was initially explored in non–nervous system tumors [1], it is now clearly appreciated to be a fundamental determinant of brain cancer biology. Similar to cancers in other organs, the brain tumor microenvironment contains endothelial cells and monocytes (macrophages and microglia). As such, pioneering studies by Judah Folkman and colleagues revealed a critical role for endothelial cells in the tumor milieu [2], leading to brain tumor therapies that focus on inhibiting vascular endothelial growth factor activity [3,4].

In addition to endothelial cells, brain tumor macrophages and microglia represent other logical targets for stroma-directed therapies. Analyses of human gliomas have revealed that 30% to 50% of the cells in these central nervous system tumors are microglia or macrophages [5–8], where the monocyte content has been associated with increasing glioma malignancy grade [9]. Moreover, numerous studies have revealed critical roles for microglia in high-grade glioma growth and progression. In these studies, microglia produce factors (gliomagens) that increase the growth and migration of glioma cells [10–12]. Importantly, pharmacological or genetic disruption of microglia function in mouse high-grade glioma models results in attenuated tumor growth and progression [13–15].

In contrast to their high-grade counterparts, less is known about the role of microglia in low-grade gliomas. The most common inherited cause of low-grade glioma is the neurofibromatosis type 1 (NF1) cancer predisposition syndrome, in which 15% to 20% of children develop pilocytic astrocytomas (PAs) involving the optic pathway [16]. Children with NF1 are born with one mutated copy of the NF1 gene and develop tumors following somatic inactivation of the remaining normal NF1 gene in astroglial progenitors [5,17]. Similar to their human counterparts, nearly all NF1+/− mice with somatic NF1 gene inactivation in neuroglial progenitors develop low-grade gliomas of the optic nerve and chiasm [18]. These resulting low-grade tumors are composed of neoplastic cells with low proliferative indices embedded within a microenvironment containing microglia and endothelial cells [18–20]. Moreover, pharmacological (minocycline treatment, JNK inhibition) or genetic (CD11b-thymidine kinase–mediated monocyte reduction or the use of Cx3cr1 knockout mice) inhibition of microglial function is sufficient to attenuate optic glioma formation and maintenance [8,21–23].

In an effort to define the molecular mechanism(s) underlying stromal maintenance of glioma growth in vivo, we sought to identify candidate gliomagens uniquely expressed in tumor-associated microglia. Whereas our previous studies employed NF1+/− microglia expanded in vitro [22], we now specifically focus on NF1+/− microglia present in the glioma as a means to discover critical glioma-maintaining factors. Building on recent advances in RNA sequencing and the analysis of low-abundance and low-quality RNA [24–27], NF1+/− microglia were isolated from control and tumor-bearing optic nerves for this large-scale discovery effort. Following secondary validation and analysis, chemokine (C–C motif) ligand 5 (Ccl5) was identified as a candidate gliomagen elaborated by tumor-associated microglia in genetically engineered NF1 mouse optic gliomas, which is also overexpressed in human PAs. Importantly, minocycline-mediated microglia inactivation decreased Ccl5 expression in vivo, whereas exogenous Ccl5 treatment increased the proliferation of NF1-deficient optic nerve astrocytes in vitro. In addition, neutralizing Ccl5 antibody administration reduced glioma growth and optic glioma–associated retinal defects in vivo. Collectively, these experimental results establish a critical role for stromal Ccl5 in the pathobiology of low-grade brain tumors.

Materials and Methods

Mice

Three independent NF1 optic glioma GEM models were used based on the timing of NF1 inactivation or the presence of additional genetic changes. The first model (NF1fl/fl; GFAP-Cre (FMC) [18]) was generated by successive breeding of NF1+/− mice with NF1fl/fl (WT) mice [28] and GFAP-Cre mice [29]. In this model, NF1 inactivation occurs in neuroglial progenitors at E14.5, whereas in the second model (FMC* [30,31]), NF1 loss occurs in neuroglial progenitors at E11.5. The third NF1 optic glioma GEM harbors Pten reduction and NF1 loss in astroglial cells [32]. NF1fl/fl; Pten−/−; GFAP-Cre (FMC) mice [33] were generated by intercrossing Pten−/−; GFAP-Cre mice [34] with NF1fl/fl; GFAP-Cre mice [29]. The resulting Pten−/−; GFAP-Cre mice were then mated with NF1fl/fl; FMPC mice to generate FMC mice. In this model, NF1 inactivation and Pten reduction occur in neuroglial progenitors at E14.5. WT and FM (NF1+/−) littermates were used as non-glioma controls (Table 1). All mice were maintained on a C57BL/6 background and used in accordance with approved animal studies protocols at the Washington University School of Medicine. Mice were euthanized at 3 months of age, and optic nerves were collected from anesthetized and Ringer’s solution–perfused mice for histological analyses, RNA expression, and fluorescence-activated cell sorting (FACS). For all in vivo experiments, mice were randomly assigned to the treatment group, and the analyses were conducted in a blinded fashion.

Minocycline Treatment

Minocycline hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS, and 50 mg/kg was administered 5 days/week for 2 weeks. FMC mice were divided into two groups: one received intraperitoneal (i.p.) injections of minocycline, whereas the other received injections of vehicle alone (sterile PBS). Each cohort contained at least four mice. After the last injection, mice were euthanized and the optic nerves collected and processed for sectioning.

Anti-Ccl5 Antibody Treatment

FMC mice were treated by i.p. injection with 250 µg of either anti-Ccl5 antibody (clone 53405; R&D Systems, Minneapolis, MN) or an IgG2A isotype-matched control antibody (R&D Systems) suspended in sterile PBS. Mice received treatment every day for 2 weeks. Each cohort contained at least five mice. After the last injection, mice were euthanized and the optic nerves collected and processed for sectioning.

Human Tissue Samples

Pathologically normal optic nerve (n = 4) and optic glioma (n = 5) tissues were obtained at autopsy from female and male patients between 3 days and 17 years of age. Tissue was embedded in paraffin, and 6-µm-thick sections were cut and processed for immunohistochemical staining. These autopsy specimens were obtained in accordance with an active and approved Human
Studies Institutional Review Board protocol at the Washington University School of Medicine.

**Primary Astrocyte Cultures**

Primary astrocyte cultures were established from the optic nerves of postnatal day 1 to 2 Nf1flox/flox pups [35]. Nf1-deficient (Nf1−/−) cultures were generated following infection with adenovirus type 5 containing Cre recombinase (University of Iowa Gene Transfer Vector Core, Iowa City, IA). To measure cell proliferation, 5 × 10⁴ astroglial cells were plated in 24-well dishes, allowed to adhere, and maintained in astrocyte growth media for 16 hours. Astrocyte cultures were then treated with either murine recombinant Ccl5 (250 ng/ml; R&D Systems) or PBS alone for up to 16 hours. The optimal cytokine concentration for the proliferation assay was predetermined using dose escalation experiments (data not shown).

**Immunocytochemistry**

Astrocytes were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Following overnight incubation with Ki67 antibodies (Abcam, Cambridge, MA), visualization was performed following incubation with Alexa Fluor 488 IgG secondary antibodies (Invitrogen, Carlsbad, CA). Cells were counterstained with DAPI. For each independent culture, at least five distinct microscopic fields were analyzed on a Nikon Eclipse TE300 fluorescence inverted microscope (Nikon, Tokyo, Japan) equipped with an optical camera (Optronics, Goleta, CA) and MetaMorph image analysis software (Molecular Devices, Dowingtown, PA).

**Immunohistochemistry**

Optic nerves were prepared for sectioning and immunostaining as previously described [36]. For paraffin section immunohistochemistry, HRP-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) were used in combination with Vectastain Elite ABC development and hematoxylin counterstaining. In the immunofluorescence detection experiments, appropriate Alexa Fluor–tagged secondary antibodies (Invitrogen) were used, followed by DAPI counterstaining. Amplification of the Brn3a and Ccl5 antibody signal was performed using a biotinylated secondary antibody, followed by HRP conjugation using Vectastain Elite ABC kit, and the fluorescent signal was amplified with Tyramide Signal Amplification (Amersham). Immunohistochemistry was performed as previously described [22].

**RNA Fluorescence In Situ Hybridization (FISH)**

FISH was performed using the QuantiGene ViewRNA kit (Affymetrix Inc., Frederick, MD) according to the manufacturer’s instructions. Conditions were optimized to include 10-minute boiling and 10-minute protease treatments. The oligonucleotide probes were commercially designed using murine Ccl5 (accession number NM_013653.3) and Cxcl13 (NM_018866.2) sequences. Images were obtained on a Nikon Eclipse TE300 fluorescence inverted microscope (Nikon) and analyzed using MetaMorph image analysis software (Molecular Devices). Individual mRNA punctae were manually counted, and the number of mRNA molecules per DAPI+ cell was calculated.

**Fluorescence-Activated Cell Sorting**

CD11b+CD45low microglia from pools of 9 to 10 optic nerves/set (FM and FMC) were collected and processed for antibody-mediated flow sorting (Supplementary Table 1) using appropriate controls for gating, as previously described [8,37]. FACS samples were sorted directly into
TRizol (Life Technologies Corporation, Carlsbad, CA) for total RNA extraction. Sorting was performed at the High-Speed Cell Sorter Core Facility at the Siteman Cancer Center, Washington University, and data were subsequently analyzed using FlowJo (Tree Star, Inc., Ashland, OR).

RNA Extraction

TRizol-chloroform extraction was used to isolate total RNA from flow-sorted microglia. Extracted RNA samples were resuspended in Ambion Nuclease-free water (Life Technologies), snap frozen, and stored at −80°C. Before cDNA library construction and quantitative real-time polymerase chain reaction (qRT-PCR), residual DNA was eliminated with the TURBO DNA-free kit (Invitrogen). RNA quality was assayed using the Agilent Eukaryotic Total RNA 6000 and quantified using the Quant-iT RNA assay kit on a Qubit Fluorometer (Life Technologies).

RNA Sequencing

The Ovation RNA-Seq method was employed for cDNA synthesis according to the manufacturer’s instructions (NuGen, San Carlos, CA). cDNA was then concentrated and suspended in 10 mM Tris-HCl (pH 8) using MiniElute spin columns (Qiagen, Valencia, CA). cDNA was quantified using the Quant-iT dsDNA HS Assay (Life Technologies Corporation), whereas the molecular weight distribution was determined using the BioAnalyzer 2100 and the Agilent DNA 7500 Chip Assay (Agilent Technologies, Santa Clara, CA). A total of 500 ng of cDNA (10ng/μl) was used for Illumina library construction with the Illumina paired-end LT indexing library was hybridized with the Agilent mouse exome reagent [40]. TopHat suite [41] with Cufflinks [42] generated biological replicates that included four samples of FM and four samples of tumor-associated microglia. Corresponding RNA-Seq paired-end reads were processed using the TopHat suite [41] with Cufflinks [42–44]. All RNA-sequencing data will be uploaded into Gene Expression Omnibus (GEO) database.

Quantitative Real-Time Polymerase Chain Reaction

The Ovation Pico WTA System V2 was employed for cDNA synthesis according to the manufacturer’s instructions (NuGen). cDNA was then purified using the MinElute Reaction Cleanup kit (Qiagen), followed by assessment of the concentration using a NanoDrop2000 spectrophotometer. qRT-PCR was performed using the Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA) with SYBR Green detection (Life Technologies Corporation). Primer sequences were designed with Primer-BLAST (NCBI http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon junctions and target known splice variants (Suppl. Table 2). The ΔΔCT method was used to calculate fold expression changes.

Human CCL5 Expression Analysis

CCL5 expression in human PAs was analyzed from the GEO dataset accession GSE44971 and GSE42656.

Statistical Analyses

All in vitro experiments were performed on independent litters, repeated at least three times, and analyzed in blinded fashion. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad, La Jolla, CA). Data were presented as mean values with SEM. Data between two groups were compared using unpaired two-tailed Student’s t tests. Data among multiple groups were compared using Kruskal-Wallis test followed by Dunn’s multiple comparison testing, with a significance level set at }P < .05. Grubbs outlier test was used to determine statistical outliers.

Results and Discussion

Optic Glioma–Associated Microglia Exhibit Morphologic Changes Suggestive of Activation

To examine microglia associated with mouse low-grade glioma, we employed three distinct models of Nf1 optic glioma. These three Nf1 murine optic glioma models differ by the timing of somatic Nf1 gene inactivation in neuroglial cells (FMC and FMC* mice; [18,30]) or the presence of additional neoplastic cell genetic changes (heterozygous Pten mutation; FMPC mice; [45]) reported in rare Nf1-associated optic pathway gliomas (OPGs) [46]. Previous studies from our laboratory have demonstrated that the tissue macrocytes in these tumors represent resident microglia (CD11b+; CD45low) with robust Cx3cr1 expression rather than bone marrow–derived macrophages (CD11b+; CD45high) [8,23]. Whereas prior studies of murine and human Nf1 optic nerve gliomas demonstrated an increased percentage of microglia in these tumors [8,18,19], confocal image analysis of these microglia within the mouse optic gliomas also revealed striking morphological changes: Nonglioma (resident) microglia in the optic nerve from either WT or Nf1+/- (FM) mice exhibited morphologies typical of ramified microglia, with elongated, fine processes that extended into their surroundings, whereas tumor-associated Nf1 +/- microglia (FMC, FMC*, and FMPC mice) harbored shorter processes that were slightly thickened or lacked processes completely (Figure 1, a and b). Similar changes in morphology were also observed in human OPG specimens relative to their normal tissue (optic nerve) counterparts (Figure 1, c and d), including one specimen from a child with Nf1.

These pronounced changes in microglial morphology have previously been interpreted as microglia “activation” [47]. As such, under normal physiological conditions, microglia have surveillance functions, with an extensive array of fine processes that constantly scan their surroundings [48]. However, in the setting of brain pathology, like Alzheimer’s or Parkinson’s disease [49], microglia undergo morphological changes that include thickening and shortening, or even retraction, of their cellular processes to assume an “amoeboid” morphology. The presence of these morphologic changes in tumor-associated Nf1 +/- microglia prompted us to specifically examine these tissue macrophages as the source of potential glioma-maintaining stroma-derived growth factors [50], distinct from those made by resting Nf1 +/- microglia within the nonneoplastic optic nerve.

Ccl5 and Cxcl13 Are Highly Expressed Glioma-Associated Microglia Transcripts

To identify potential gliomagens produced by tumor-associated microglia, we initiated a large-scale RNA-sequencing discovery effort. We were specifically interested in transcripts unique to Nf1+-/ microglia within optic gliomas relative to those found in Nf1+/- microglia within the normal (non-neoplastic) optic nerve. Because the mouse optic nerve is composed of fewer than 10% microglia [23] and only ~2500 total cells per nerve can be recovered by FACS after processing, we pooled at least nine 3-month-old mice per genotype to process, we pooled at least nine 3-month-old mice per genotype to
associated with FACS separation, RNA isolated from microglia under these conditions is in low abundance and frequently of low quality (Figure 2a), necessitating optimization of RNA isolation and analysis methods [24–27]. To circumvent these issues, we employed a combination of exome capture enrichment and Illumina RNA sequencing (cDNA-Capture sequencing) on microglia isolated from optic glioma–bearing FMC mice and Nf1 +/- non–tumor-bearing (FM) mice. Samples were analyzed using the Cufflinks platform [42–44] to calculate the differential expression of genes from each pool (FMC and FM microglia). We further narrowed the candidate list to transcripts whose predicted protein products were either secreted or cell surface associated and also exhibited at least a 10-fold

Figure 1. Human and murine optic glioma–associated microglia exhibit morphological changes. (a) Whereas microglia from normal (WT and FM; n = 5 genotype) mouse optic nerves were ramified cells with long Iba1+ processes, glioma-associated (FMC, FMC*, and FMPC; n = 5 genotype) microglia were amoeboid-shaped monocytic cells with shorter processes. Scale bar, 100 µm. The differences in microglial process lengths were quantified in panel b. Similar to the mouse tumors, human optic glioma–associated microglia also harbored short or no processes (c). The differences in microglial process lengths were quantified in panel d. DAPI (blue) was used as a counterstain to identify all cells in the sections. Scale bar, 50 µm. The dotted areas denote the regions from which the high-power images in the right panels derived. Each error bar represents the mean ± SEM. Asterisks denote statistically significant differences.
change in expression (six genes). Of these six candidates, only Ccl5 and Cxcl13 were independently validated by RNA FISH and qRT-PCR as potential optic glioma–associated microglia gliomagens (Figure 2b). Other transcripts previously identified in studies using either high-grade glioma-associated or non–tumor-associated Nf1+/− brain microglia maintained in vitro, including interleukin-6, interleukin-10 [51,52], stem cell factor [53], transforming growth factor beta [54], hyaluronidase, hepatocyte growth factor [55], pleiotrophin, jagged-1, and insulin-like growth factor–1 [22], were not uniquely expressed by optic glioma–associated microglia relative to their nonneoplastic Nf1+/− optic nerve microglia counterparts.

Specifically, RNA FISH demonstrated increased Cxcl13 and Ccl5 transcripts in optic glioma (FMC; n = 3) compared with non–tumor-bearing (FM; n = 6) optic nerves. Representative images are shown with insets of cells containing mRNA molecules (red). DAPI (blue) was used as a counterstain to identify all cells in the sections. Scale bar, 50 μm. (d) SYBR-Green qRT-PCR of independently generated FACS-isolated FMC microglia revealed increased Cxcl13 and Ccl5 expression relative to FM microglia. Each error bar represents the mean ± SEM. Asterisks denote statistically significant differences: (*) P = .0312.

Minocycline Treatment Reduces Optic Glioma Microglial Ccl5 Expression

Previous studies have demonstrated that minocycline inactivation of microglia reduced Nf1 optic glioma proliferation in vivo [22]. To provide further support for a role of Ccl5 and Cxcl13 in mouse Nf1 optic glioma growth, FMC mice (n = 4 mice/group) were i.p. injected with either minocycline or vehicle over a 2-week period, and the optic nerves were analyzed by immunohistochemistry and RNA FISH. Following minocycline treatment, the morphology of microglia (thin, long, ramified processes) was restored to that observed in WT or FM mice (Figure 3a). Importantly, whereas minocycline treatment had no effect on Cxcl13 expression, Ccl5 expression was decreased to control
These observations suggest that Ccl5, but not Cxcl13, is particularly worthy of further exploration as a potential tumor-associated microglia gliomagen.

Ccl5 Is Important for Nf1 Optic Glioma Growth

Because only Ccl5 expression was decreased following minocycline treatment, we focused subsequent experiments on this high-priority candidate. First, we examined Ccl5 expression at the protein level in FM and FMC optic nerves. Relative to their nonneoplastic counterparts, there was an 8.4-fold increase in the percentage of Ccl5+ cells in the optic gliomas (Figure 4a). Moreover, nearly all of the Ccl5+ cells were Iba1+ cells (microglia) in these tumors by double-labeling immunofluorescent microscopy (Figure 4b).

Second, because symptomatic Nf1-associated optic gliomas are commonly treated without a prior tissue diagnosis (biopsy), we leveraged available transcriptomal human PA data sets (GSE42556 [57] and GSE44971 [58]). Analysis of the GSE42556 specimens revealed increased CCL5 expression (all three independent CCL5 probe sets) in sporadic PAs (n = 46) relative to normal brain controls (n = 9), including three additional tumors from individuals with NF1 (Figure 4c). Similarly, analysis of the GSE44971 specimens revealed increased CCL5 expression (both of the two independent CCL5 probe sets) in the sporadic PAs (n = 14) relative to normal brain controls (n = 16) (Figure 4d). In contrast, no increase in CXCL13 expression was observed in the PA tumors from either data set (data not shown), further underscoring the importance of CCL5 in low-grade glioma.

Third, to determine whether Ccl5 has the capacity to increase astrocyte growth in vitro, we employed Nf1-deficient optic nerve glia cultures following acute Nf1 gene inactivation (adenovirus-mediated Cre transduction). Because these cultures contain 70% NG2+ cells and 30% GFAP+ cells, in which only the GFAP+ astrocytes
hyperproliferate following \( Nf1 \) gene inactivation \[59\], we analyzed the proliferation of optic nerve astroglial cells using Ki67 labeling in vitro. Following treatment with murine recombinant \( \text{Ccl5} \) (250 ng/ml), there was a 1.5-fold increase in astrocyte proliferation (Figure 5), demonstrating that \( \text{Ccl5} \) is sufficient to increase \( Nf1 \)-deficient astrocyte growth.
Fourth, to establish that increased microglial Ccl5 expression is necessary for Nf1 optic glioma growth, we sought to inhibit Ccl5 function in vivo. Because there are few selective small molecule inhibitors of Ccl5 that cross the blood-brain barrier, we initially employed MET-RANTES, an amino-terminal-modified methionylated Ccl5 derivative [60, 61] originally identified to competitively bind to Ccr1 and Ccr5 and inhibit the signaling pathways activated by Ccl5 [61]. In other experimental models of nervous system disease, mice treated with MET-RANTES had less severe clinical symptoms and reduced inflammation [62–64]. However, MET-RANTES–treated mice exhibited increased Nf1 optic glioma growth and more Iba1+ microglia compared with PBS-treated mice (Supplementary Figure 2). The paradoxical effect of MET-RANTES treatment is likely attributable to its function as a partial agonist [65], which prompted us to explore other methods to target Ccl5.

Previous preclinical studies have used anti-Ccl5 neutralizing antibodies to interfere with Ccl5 function and attenuate tumor growth in vivo [66, 67]. Specifically, treatment with Ccl5 antibody blocked leukocyte adhesion and infiltration in experimental mouse models of multiple sclerosis [68, 69]. Based on these studies, 3-month-old FMC mice were treated with either anti-Ccl5 (250 μg) or IgG isotype control antibodies i.p. daily for 2 weeks (n = 5 mice/group). Following the completion of treatment, mice were injected with BrdU (50 mg/kg), and the optic nerves were removed 3 hours later for analysis (Figure 6a). Anti-Ccl5 treatment resulted in a 9.8-fold reduction in Nf1 optic glioma proliferation (BrdU+ cells) relative to IgG control–treated mice in vivo (P < .0001; Figure 6b). Interestingly, consistent with the known chemotactant properties of Ccl5 [70–72], anti-Ccl5 treatment also reduced the number of Iba1+ microglia into these tumors in vivo (Figure 6a).

Because optic gliomas cause clinical morbidity in children with NF1 as a result of reduced visual function [73], the impact of anti-Ccl5 treatment on optic glioma–induced retinal dysfunction was assessed. In Nf1 optic glioma mice, visual impairment occurs following retinal ganglion cell (RGC) apoptosis and loss [33]. Following anti-Ccl5 treatment, there were a 2.8-fold decrease in the percent of apoptotic (%TUNEL+) cells and a 1.78-fold increase in RGCs (Brn3a+ cells; Figure 6d) relative to IgG-treated controls. Collectively, these findings demonstrate that microglia-produced Ccl5 is both necessary and sufficient to increase Nf1-deficient astrocyte proliferation relevant to Nf1 optic glioma maintenance and that inhibiting Ccl5 function reduces retinal pathology in the setting of murine Nf1 optic glioma.

The identification of Ccl5 as a candidate gliomagen in Nf1 murine optic glioma suggests a new stromal target for therapeutic drug design. Ccl5 is also known as RANTES (regulated upon activation, normal T-cell–expressed and secreted), where it was originally identified as an inducer of leukocyte recruitment to sites of inflammation [74]. Ccl5 induces leukocyte migration by binding to three distinct seven-transmembrane G-protein–coupled receptors [75–77]. In the setting of brain pathology, increased CCL5 expression in astrocytes and microglia has been reported following viral infection [78–81], where its inhibition results in decreased leukocyte adhesion within the microcirculation of infected mice [82]. Importantly, mice lacking Ccr5 expression exhibit reduced neuronal injury in responses to HIV-1 infection [83], supporting a general role for CCL5 signaling in neurologic disease.

One previous study examined CCL5 in malignant glioma, demonstrating increased expression of CCL5 and its receptors (CCR1, CCR5) in the murine high-grade GL261 astrocytoma cell line [84]. In contrast, CCL5 function in low-grade gliomas has not been explored, and the impact of CCL5 function on glioma biology remains unclear. Whereas little is known about CCL5 in brain tumors, increased CCL5 expression has been reported in lung, prostate, melanoma, colorectal, and breast cancer [67, 85–88], where a positive correlation between increased CCL5 expression and disease progression was identified [89, 90]. In addition, CCL5–expressing melanoma cells form increasingly aggressive tumors in a concentration-dependent fashion [91], and Ccl5 increases the growth of breast cancer cells in vitro [92]. Finally, blocking Ccl5 receptor binding using neutralizing antibodies or siRNA knockdown reduces tumor growth in experimental models of pancreatic adenocarcinoma [93], colon cancer [67], and gastric cancer [94].

In the current study, we provide experimental evidence for a critical role for Ccl5 in the maintenance of murine Nf1 optic glioma. Following inhibition of Ccl5 with neutralizing antibodies, there were

Figure 5. Ccl5 increases optic nerve astrocyte proliferation in vitro. Following incubation with 250 ng of murine recombinant Ccl5, there was an increase in Nf1-deficient astrocyte proliferation (Ki67 labeling) relative to vehicle treatment in vitro. Representative Ki67+ proliferating cells (green) and DAPI+ nuclei (blue) are shown. Arrowheads indicate Ki67-positive cells. Scale bar, 100 μm. Each error bar represents mean ± SEM. Asterisks denote statistically significant differences.
reduced tumor proliferation and attenuated retinal pathology. In addition, Ccl5 inhibition resulted in decreased microglia within the optic glioma, suggesting that Ccl5 may be a key stromal determinant of tumor growth and associated optic nerve damage as well as monocyte recruitment to the glioma. Although we specifically chose to directly inhibit Ccl5 function, additional preclinical investigation could involve the use of Ccl5 receptor antagonists. It should be recognized that Ccl5 can bind to one of three receptors (Ccr1, Ccr3, and Ccr5) to promote cell growth [95]. However, only Ccr1 and Ccr5 are expressed by optic nerve astrocytes (data not shown), complicating the decision regarding which Ccl5 receptor to inhibit. In this regard, Ccr5 antagonism using Maraviroc [96] had no effect on Nf1 optic glioma proliferation as measured by BrdU incorporation and (c) the percentage of Iba1+ microglia. Scale bar, 100 μm. (d) Following anti-Ccl5 treatment, TUNEL staining (n = 5 mice/group; top panels) and Brn3a immunofluorescence (n = 5 mice/group; bottom panels) revealed decreased retinal cell apoptosis (%TUNEL+ cells) and increased RGC numbers (Brn3a+ cells) relative to IgG-treated controls. Representative individual TUNEL+ and Brn3a+ cells within the ganglion cell layer are shown in the insets. Quantification is represented in the adjacent graphs. Scale bar, 50 μm. Each error bar represents the mean ± SEM. Asterisks denote statistically significant differences. INL, inner nuclear layer; GCL, ganglion cell layer; ONL, outer nuclear layer.

Figure 6. Ccl5 is necessary for murine Nf1 optic glioma growth in vivo. (a) At 3 months of age, FMC mice received i.p. injections of either Ccl5 neutralizing antibodies (n = 5 mice) or an IgG2A isotype-matched control antibody (n = 5 mice) every day for 2 weeks. Three hours before euthanasia, mice were i.p. injected with 50 mg/kg of BrdU. Ccl5 treatment decreased (b) murine Nf1 optic glioma proliferation as measured by BrdU incorporation and (c) the percentage of Iba1+ microglia. Scale bar, 100 μm. (d) Following anti-Ccl5 treatment, TUNEL staining (n = 5 mice/group; top panels) and Brn3a immunofluorescence (n = 5 mice/group; bottom panels) revealed decreased retinal cell apoptosis (%TUNEL+ cells) and increased RGC numbers (Brn3a+ cells) relative to IgG-treated controls. Representative individual TUNEL+ and Brn3a+ cells within the ganglion cell layer are shown in the insets. Quantification is represented in the adjacent graphs. Scale bar, 50 μm. Each error bar represents the mean ± SEM. Asterisks denote statistically significant differences. INL, inner nuclear layer; GCL, ganglion cell layer; ONL, outer nuclear layer.
role of stroma-derived chemokines in glioma biology. In this regard, CXCL12 is produced by both microglia [97] and endothelial cells [98,99], where it can dually act to further attract additional microglia as well as independently stimulate tumor growth. In addition, chemokines can also be produced by glioma cells to direct microglia migration and recruitment. For example, CX3CL1 acting on the microglial CX3CR1 receptor is critical for microglia infiltration and tumor formation in mouse Nf1 optic glioma strains [23]. Similarly, colony-stimulating factor 1 (CSF-1) produced by mouse glioblastoma cells attracts microglia through the CSF-1 receptor to further increase glioma growth [100], such that attenuation of this CSF-1/Csf1 receptor axis reduced murine glioblastoma growth in vivo [15]. Collectively, these studies suggest that future brain tumor therapies might target the unique interplay between stromal cells and cancer cells important for maintaining this specialized ecosystem.

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Conflict of Interest

The authors declare no conflicts of interest.

Authors’ Contributions

W. W. P., V. M., M. H. E., E. R. M., and D. H. G. jointly supervised research. A. C. S., W. W. P., E. R. M., and D. H. G. conceived and designed the experiments. J. W., T. W., V. M., and D. H. contributed to experimental design. A. C. S., W. W. P., P. J. C., K. Y. K., and J. A. T. performed the experiments. A. C. S., W. W. P., J. A. T., J. W., T. W., V. M., M. G., and O. L. G. analyzed the data. V. M., M. H. E., E. R. M., and D. H. G. contributed reagents/materials/analysis tools. A. C. S., W. W. P., K. Y. K., J. A. T., J. W., T. W., V. M., M. G., and O. L. G. contributed to the preparation of the manuscript. A. C. S., W. W. P., and D. H. G. wrote the manuscript. All authors edited and approved the manuscript.

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