RNA sequencing of tumor-associated microglia reveals Ccl5 as a stromal chemokine critical for neurofibromatosis-1 glioma growth

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

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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 glomas 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 gloma 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.

Neoplasia (2015) 17, 776-788

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...
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 (NF1flox/flox; GFAP-Cre (FM) [18]) was generated by successive breeding of NF1flox/flox (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 (FM*) [30,31], NF1 loss occurs in neuroglial progenitors at E11.5. The third NF1 optic glioma GEM model harbors Pten reduction and NF1 loss in astroglial cells [32]. NF1flox/flox; Ptenflklox/−; GFAP-Cre (FMPC) mice [33] were generated by intercrossing Ptenflklox/− mice [34] with NF1flox/flox; GFAP-Cre mice [29]. The resulting Ptenflklox/−; NF1flox/flox; GFAP-Cre mice were then mated with NF1flox/flox mice to generate FMPC 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 × 10^4 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) and analyzed using 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 Bm3a 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 (PerkinElmer) according to the manufacturer’s instructions. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the ApopTag Plus in situ apoptosis fluorescent detection kit (Millipore, Billerica, MA) according to the manufacturer’s recommendations. Images were subsequently acquired on a Nikon Eclipse TE300 fluorescence inverted microscope or a Nikon Eclipse E600 microscope equipped with an optical camera (Leica, Buffalo Grove, IL) and Leica LAS EZ image analysis software (Leica).

Alternatively, optic nerves were processed for O.C.T. (Tissue-Tek, Miles, Elkhart, IN) embedding (frozen sections). Immunofluorescence labeling was performed after blocking in PBS containing 3% normal donkey serum (Jackson Immunoresearch Labs, Westgrove, PA), 1% cold water fish gelatin (Sigma-Aldrich), and 0.1% Triton X-100 for 1 hour at room temperature before incubation with appropriate antibodies (Supplementary Table 1) in 10% normal donkey serum for 16 hours at 4°C. Fluorescence-conjugated secondary antibodies (1:100 dilution) were applied for 4 hours at 4°C. Images were acquired on a Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan).

The number of lineage antibody-positive, Ki67+, or TUNEL+ cells was quantitated as a percentage of total cells (DAPI+ cells or nuclei).

Microglial morphology was analyzed using ImageJ (version 1.48, NIH software. The length of the microglial processes was defined as the distance between the nucleus and the tip of an extended process as identified by Iba1 immunostaining (Supplementary Figure 1).

**In vivo BrdU Labeling and Immunohistological Analysis**

IgG and anti-Ccl5–treated animals were injected with 50 mg/kg of BrdU (Sigma-Aldrich). Three hours after BrdU injection, animals were transcardially perfused with ice-cold Ringer’s solution and fixed with 4% paraformaldehyde. The optic nerves were dissected, fixed, and processed for paraffin sectioning, and BrdU immunostaining was performed as described previously [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_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

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**Table 1. Genetically Engineered Nf1 Mouse Models.**

<table>
<thead>
<tr>
<th>Short Form</th>
<th>Genotype</th>
<th>Cell Type</th>
<th>Description</th>
<th>Time Point</th>
<th>CNS Abnormality</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Nf1flox/+</td>
<td>Every cell</td>
<td>Exons 31 and 32 of the Nf1 gene flanked by loxp sites</td>
<td>None</td>
<td>None</td>
<td>Zhu et al., 2001</td>
</tr>
<tr>
<td>FM</td>
<td>Nf1flox/+</td>
<td>Every cell</td>
<td>Reduced Nf1 gene expression</td>
<td>E14.5</td>
<td>None</td>
<td>Bajenaru et al., 2003</td>
</tr>
<tr>
<td>FMC</td>
<td>Nf1flox/+</td>
<td>Neuroglial progenitors</td>
<td>Complete Nf1 loss</td>
<td>E11.5</td>
<td>Optic glioma</td>
<td>Hegedus et al., 2008</td>
</tr>
<tr>
<td>FMC*</td>
<td>Nf1flox/+</td>
<td>Surrounding cells</td>
<td>Complete Nf1 loss</td>
<td>E14.5</td>
<td>Optic glioma</td>
<td>Kaul et al., 2014</td>
</tr>
<tr>
<td>FMPC</td>
<td>Nf1flox/+</td>
<td>Neuroglial progenitors</td>
<td>Reduced Nf1 expression</td>
<td>E14.5</td>
<td>Optic glioma</td>
<td>Kaul et al., 2014</td>
</tr>
</tbody>
</table>
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 Nucleic-acid-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 MinElute 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 protocol as previously published [38,39]. Briefly, 500 ng of each 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 (NCBIhttp://www.ncbi.nlm.nih.gov/tools/primer-blast/ ) to span 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 "amoeboid" morphology. The presence of these morphologic changes in tumor-associated NFI +/- 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 NFI +/- microglia within the nonneoplastic optic nerve.

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 Nfi optic glioma. These three Nfi murine optic glioma models differ by the timing of somatic Nfi 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 NFI-associated optic pathway gliomas (OPGs) [46]. Previous studies from our laboratory have demonstrated that the tissue monocytes 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 NFI 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 Nfi +/- (FM) mice exhibited morphologies typical of ramified microglia, with elongated, fine processes that extended into their surroundings, whereas tumor-associated Nfi +/- 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 NFI +/- 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 NFI +/- microglia within the nonneoplastic optic nerve.

Ccl5 and Cxcl13 Are Highly Expressed Glioma-Associated Microglia Transcripts

To identify potential gliomas produced by tumor-associated microglia, we initiated a large-scale RNA-sequencing discovery effort. We were specifically interested in transcripts unique to Nfi +/- microglia within optic gliomas relative to those found in Nfi +/- 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 obtain sufficient numbers of cells for FACS and RNA isolation. Because of the extended processing times (~8 hours) required, the small amounts of starting material, and the inherent cell loss
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 ameboid-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 expression in murine 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.

**Figure 2.** Cxcl13 and Ccl5 expression are increased in murine optic glioma. (a) CD11b+ CD45low microglia from pools of 9 to 10 optic nerves/set (FM and FMC) were collected and processed for antibody-mediated flow sorting. (b) Six transcripts were identified that represented predicted secreted or cell surface–associated proteins with at least a 10-fold overexpression in FMC relative to FM microglia (six genes). FC, fold change (FMC/FM). N.D., not done. (c) 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 3d). Importantly, whereas minocycline treatment had no effect on Cxcl13 expression, Ccl5 expression was decreased to control
levels (Figure 3b). 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 \textit{Nf1} gene inactivation \cite{59}, we analyzed the proliferation of optic nerve astroglial cells using Ki67 labeling \textit{in vitro}. Following treatment with murine recombinant Ccl5 (250 ng/ml), there was a 1.5-fold increase in astrocyte proliferation (Figure 5), demonstrating that Ccl5 is \textit{sufficient} to increase \textit{Nf1}-deficient astrocyte growth.

Figure 4. Ccl5 expression is increased in murine OPGs and human PAs. (a) Immunofluorescence of murine optic nerve tissue reveals increased Ccl5 (green) protein expression in \textit{Nf1} optic glioma specimens (FMC, \(n = 5\)) relative to control optic nerve tissue (FM, \(n = 5\)). Quantification is represented in the adjacent graph. Each error bar represents the mean \(\pm\) SEM. Asterisks denote statistically significant differences: (** \(P = .0079\)). (b) Double labeling of Ccl5 and Iba1 reveals that the chemokine Ccl5 is expressed in Iba1\(^{+}\) cells (microglia). Scale bar, 50 \(\mu\)m. DAPI (blue) was used as a counterstain to identify all cells in the sections. Representative images are shown, with insets demonstrating immunopositive cells (arrows). (c) In the GSE44971 GEO dataset, \textit{CCL5} RNA expression in human PAs (\(n = 46\) sporadic tumors and 3 from individuals with NF1) was increased for all three \textit{CCL5} probe sets relative to normal brain (\(n = 9\)). The white bars denote normal brain samples, the black bars denote the sporadic PA specimens, and the hatched bars denote the NF1-associated PAs. Each error bar represents the mean \(\pm\) SD. (**) \(P = .0015\) using a one-way analysis of variance with Bonferroni posttest correction. (d) In the GSE42656 GEO dataset, \textit{CCL5} RNA expression was also increased for both \textit{CCL5} probe sets. The white bars denote normal brain samples (\(n = 16\)), whereas the black bars denote the PA specimens (\(n = 14\)). Each error bar represents the mean \(\pm\) SD. (*** \(P < .0001\) using a two-way analysis of variance with Bonferroni post-test correction.)
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-Cd5 neutralizing antibodies to interfere with Cdl5 function and attenuate tumor growth in vivo [66,67]. Specifically, treatment with Cd5 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-Cdl5 (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-Cdl5 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 chemoattractant properties of Cd5 [70–72], anti-Cd5 treatment also reduced the number of Iba1+ microglia into these tumors in vivo (Figure 6c).

Because optic gliomas cause clinical morbidity in children with NF1 as a result of reduced visual function [73], the impact of anti-Cd5 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-Cd5 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 Cdl5 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 Cd5 as a candidate gliomagen in Nf1 murine optic glioma suggests a new stromal target for therapeutic drug design. Cd5 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 Cd5 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 Cd5 in the maintenance of murine Nf1 optic glioma. Following inhibition of Cd5 with neutralizing antibodies, there were
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 (data not shown), whereas MET-RANTES increased Nf1 optic glioma growth. Based on these findings, future preclinical studies will need to carefully consider the specific effects of each Ccl5 inhibitor and the engagement of its specific cognate receptors.

In summary, the observations in this report not only underscore the importance of microglia in the glioma microenvironment in maintaining tumor growth but also expand our understanding of the

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/CSF-1 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

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