**Abstract**

*KIAA1549-BRAF* is the most frequently identified genetic mutation in sporadic pilocytic astrocytoma (PA), creating a fusion BRAF (f-BRAF) protein with increased BRAF activity. *Fusion-BRAF*-expressing neural stem cells (NSCs) exhibit increased cell growth and can generate glioma-like lesions following injection into the cerebella of naive mice. Increased Iba1+ monocyte (microglia) infiltration is associated with murine *f-BRAF*-expressing NSC-induced glioma-like lesion formation, suggesting that *f-BRAF*-expressing NSCs attract microglia to establish a microenvironment supportive of tumorigenesis. Herein, we identify Ccl2 as the chemokine produced by *f-BRAF*-expressing NSCs, which is critical for creating a permissive stroma for gliomagenesis. In addition, *f-BRAF* regulation of Ccl2 production operates in an ERK- and NFκB-dependent manner in cerebellar NSCs. Finally, Ccr2-mediated microglia recruitment is required for glioma-like lesion formation in vivo, as tumor do not form in *Ccr2*-deficient mice following *f-BRAF*-expressing NSC injection. Collectively, these results demonstrate that *f-BRAF* expression creates a supportive tumor microenvironment through NFκB-mediated Ccl2 production and microglia recruitment.

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**Introduction**

Low-grade gliomas (LGGs) represent the most common central nervous system (CNS) tumor occurring in children, with pilocytic astrocytoma (PAs) being the most frequently encountered benign neoplasm in this age group [1]. The majority of pediatric LGGs are caused by tandem duplications involving the *BRAF* gene (e.g., *KIAA1549:BRAF* rearrangement), which typifies sporadic PAs arising in the cerebellum [2–4]. Based on these observations, we previously demonstrated that ectopic *KIAA1549:BRAF* expression in mouse cerebellar neural stem cells (NSCs) is sufficient to generate glioma-like lesions following implantation in the naive murine cerebellum [5]. In addition, increased *KIAA1549:BRAF*-driven NSC proliferation is mediated through hyperactivation of the MEK/ERK signaling pathway [5].

Similar to low-grade gliomas arising in children with the Neurofibromatosis type 1 (NF1) cancer predisposition syndrome, these glial tumors are highly dependent on their local microenvironment, and in experimental model systems, are controlled by non-neoplastic stromal cells. Prior studies using *Nf1* genetically engineered mouse strains have revealed that brain monocytes (microglia) are the driving stromal cell type in these tumors, such that their genetic or pharmacologic inhibition attenuates tumor formation and growth [6–8]. Importantly, monocytes can comprise as many as 30–50% of the total number of cells in PAs [9,10]. The presence of these monocytes suggests that the recruitment and activation of microglia and macrophages represent key steps in glioma formation and maintenance.

In order to define the role of microglia in sporadic *KIAA1549:BRAF* (f-BRAF)-driven low-grade glioma, we leveraged converging *in vitro* and *in vivo* approaches to demonstrate that *KIAA1549:BRAF*...
positively regulates cerebellar NSC Ccl2 expression through ERK-dependent NFκB activation. The importance of Ccl2 to tumorigenesis was further underscored by the failure of low-grade glioma-like lesions to form following the implantation of f-BRAF-expressing NSCs into Ccr2−/− mice.

Materials and Methods

Mice

The **KIAA1549:BRAF** conditional transgenic mouse strain were generated as previously described [11]. Ccr2−/−RFP (Keiko Hirose, Washington University) were intercrossed to generate Ccr2−/−/Lox-STOP-Lox-**KIAA1549:BRAF** transgenic mice [11]. pup to establish primary neural stem cell (NSC) cultures [12]. Control and f-BRAF-expressing NSCs were generated following infection with Adenovirus type 5 (Ad5) containing β-galactosidase (Ad5-LacZ) or Cre recombinase (Ad5-Cre) (University of Iowa Gene Transfer Vector Core, Iowa City). Ectopic f-BRAF expression in NSCs was also generated through retrovirus infection (Peter Collins, University of Cambridge). The constructs used for retroviral infection were pBABE-puro **KIAA1549:BRAF** and pBABE-puro. f-BRAF-expressing cells were confirmed by RNA RT-PCR [13] using the following primer set: 5′ TCTCGCTGAGGT 3′ and 5′ GAATGGGTCCAGACATACATT 3′.

**Lentivirus Infection**

Lentivirus transduction was described previously [14]. Briefly, shRNA-Cd2 shRNA-1 (5′ GAATGTTAAGGTTACCGGTA 3′), shRNA-Cd2 shRNA-2 (5′ GAATGGTTACCACTATTTT 3′), and LacZ shRNA (5′ CGCTCATACCGATAACGGATT 3′) in the pLPKuro plasmid were individually co-transfected with pMDLg/pRRE, pRSV-REV and pCMV-VSV-G plasmids into HEK293T cells using the FuGENE HD transfection reagent (Roche). pLKpuro plasmid were individually co-transfected with pMDLg/pRRE, pRSV-REV and pCMV-VSV-G plasmids into HEK293T cells using the FuGENE HD transfection reagent (Roche). pLKpuro plasmid were individually co-transfected with pMDLg/pRRE, pRSV-REV and pCMV-VSV-G plasmids into HEK293T cells using the FuGENE HD transfection reagent (Roche). 

**Intracranial Injections**

Four-week-old wild-type C57BL/6 or Ccr2-deficient male mice were anesthetized with an intraperitoneal (i.p.) injection of 100 mg/kg ketamine and 6 mg/kg xylazine. NSCs (5×10^5 cells) in 2 μl of PBS were injected into the cerebellum using a Hamilton syringe and a stereotaxic apparatus (coordinates = posterior 5.8 mm from the bregma, lateral 0.7 mm [right], and depth 2.5 mm from dura mater). Mice were euthanized 6 months later.

**Immunohistochemistry**

Paraffin sections were processed as previously reported [12] prior to staining with appropriate primary antibodies (Vector Laboratories) in combination with Vectastain Elite ABC development and hematoxylin counterstaining.

**Real-Time qRT-PCR**

RNA was purified using the RNeasy Plus Mini Kit (Qiagen) prior to treatment with (10 μg/ml DNase I recombinase (Roche). The DNase was then heat inactivated with EDTA chelation. RNA was reverse transcribed into cDNA using the Omniscript RT kit (Qiagen). Real-time qRT-PCR was performed as previously described [15] using mouse- or human-specific primers (Supplemental Table 2). For each gene, the ΔΔCT values were calculated, where H3f3a (or H3F3A for human specimens) was used as an internal control. Chemokines and Receptors RT2 profiler PCR array (PAMM-022Z; Qiagen) analyses were performed according to the manufacturer’s recommendations.

**Human Pilocytic Astrocytoma (PA) Cell Lines**

Following Institutional Review Board approval (Protocol #2016-014), tumor specimens were procured by C.K. and C.A.W. during standard surgical resection by R.M.L. at Dayton Children’s Hospital, after necessary tissues were submitted for pathologic diagnosis. Parental consent and, when applicable, patient assent was obtained prior to collection. Fresh tissues for culture were prepared as 50 to 200-mg specimens, placed directly into culture medium consisting of Dulbecco’s Minimum Essential Media with F12, 10% heat-inactivated fetal bovine serum (FBS), and 100 μg/ml Primocin™ (Invivogen, Inc.), and transported on wet ice to a sterile hood. Tumors were minced and digested with trypsin and collagenase IV at 37 °C for 30 minutes, and then inactivated with FBS. Cells were then centrifuged at 1000 rpm for 3 minutes, resuspended in culture medium, and added to 100-mm tissue culture plates. Adherent cells were passaged when 70–80% confluent. Prior to transport, cells were removed with trypsin digestion, followed by FBS inactivation, and centrifuged at 1000 rpm for 3 minutes. Cell pellets were shipped on dry ice to Washington University for further processing and analysis. Clinical and BRAF mutation information is included in Supplemental Table S3. Normal human astrocytes (NHA cells; ScienCell Research Laboratories, Inc.) were grown according to the manufacturer’s recommendations.

**Western Blotting**

Cell pellets were lysed in RIPA lysis buffer supplemented with proteinase and phosphatase inhibitors. Protein samples were separated by SDS-PAGE, and then transferred onto Immobilon membranes. Membranes were blocked in Tris-buffered saline 0.1% Tween 20 (TBST) with 5% non-fat dry milk, and incubated with the primary antibodies listed in Supplemental Table 1. Antibodies were diluted in blocking buffer or 5% BSA TBST overnight at 4°C, and horseradish peroxidase conjugated secondary antibodies were added for 1 hour at room temperature prior to chemiluminescence development.

**ELISA**

Conditioned medium (CM), was collected from control and f-BRAF-expressing NSCs, and the levels of CCL2 determined using the Mouse/Rat or Human CCL2/JE/MCP-1 Quantikine ELISA kit (R&D). The number of cells were counted for each condition and used for normalization.

**Results and Discussion**

Since increased monocyte (microglia and macrophage) infiltration is associated with f-BRAF-expressing pilocytic astrocytomas in children [7,9,16] and f-BRAF-expressing NSC glioma-like lesion formation in
**Figure 1.** f-BRAF-expressing cerebellar NSCs produce Ccl2. (A) GFAP-immunoreactive cells are present at the injection sites of control and f-BRAF NSC-injected wild-type mice. Injected sites with tumors also contain GFP-positive f-BRAF-expressing cells. Increased Iba1+ cells were detected in f-BRAF-induced lesions. (B) f-BRAF transgene expression was confirmed by RT-PCR in KIAA1549:BRAF cerebellar NSCs infected with Ad5-Cre. f-BRAF-expressing cerebellar NSCs have increased proliferation relative to controls. (C) Increased ERK and S6 phosphorylation was observed following f-BRAF expression in cerebellar NSCs. (D) Chemokine antibody array revealed increased Cxcl10, Ccl2, Cxcl1 and Cxcl5 expression in the culture medium (CM) of f-BRAF-expressing cerebellar NSCs relative to controls. Boxes denote the increased candidate chemokines. (E) Quantitative analysis of increased chemokines in f-BRAF-expressing cerebellar NSCs using a chemokine PCR array. The mRNA level fold change (FC) for the indicated chemokines are shown in the Table. (F) Ccl2 knockdown using two independently generated Ccl2 shRNAs demonstrates decreased Ccl2 mRNA in f-BRAF-expressing cerebellar NSCs (left). Cxcl10 mRNA levels were decreased in Ccl2 knockdown f-BRAF-expressing cerebellar NSCs (right).
mice [5], we sought to determine whether f-BRAF induces chemokine expression to recruit Lba1+ monocytes to the tumor site (Figure 1A). To induce f-BRAF expression in the presumed cells of origin for cerebellar low-grade glioma, primary cerebellar NSCs were generated from PN2 conditional knock-in KIAA1549:BRAF (f-BRAF) mice [11]. Following adenovirus infection, f-BRAF expression was only induced in Ad5-Cre-infected NSCs, but not in control Ad5-LacZ-infected cerebellar NSCs (Figure 1B). Similar results were also obtained following infection with a retrovirus containing the KIAA1549:BRAF transgene (data not shown), and these two approaches were used interchangeably. Similar to our previous studies using these methods [5,11], f-BRAF-expressing cerebellar NSCs exhibited elevated ERK activation (ERKTh202/Tyr204 phosphorylation) and increased phosphorylation (activation) of ribosomal S6 protein (Ser240/244) (Figure 1C). In addition, f-BRAF-expressing cerebellum NSCs proliferated faster than infected controls (Figure 1B). Consistent with previous reports [5,11,17–19], f-BRAF induces low-grade glioma formation and growth through increased activation of B-Raf downstream effectors, including MEK/ERK and mTOR.

To identify potential chemokines produced by f-BRAF-expressing cerebellar NSCs, we employed a commercial chemokine protein array. Since chemokines are typically secreted paracrine factors, we used conditioned medium (CM) from f-BRAF-expressing and wild type cerebellar (control) NSCs (Figure 1D). In addition, we analyzed chemokine mRNA expression using a commercial RT profiler PCR array (Figure 1E). Using both methods, four potential chemokines were identified (Cxc1l, Cxc15, Cxcl10, Ccl2) at the protein and mRNA levels. However, only Ccl2 and Cxcl1 exhibited >3-fold increases. For this reason, we focused on Ccl2 and Cxcl1.

First, we sought to determine whether Ccl2 regulated Cxcl1 expression, and therefore functioned as the key chemokine to regulate monocyte attraction. By using two independently generated Ccl2 shRNA constructs, we found that Ccl2 knockdown reduced Cxcl1 expression in f-BRAF-expressing cerebellar NSCs (Figure 1F), thus positioning Ccl2 as the master regulator worthy of further investigation.

Second, we leveraged the online NCBI GEO database, which contains two separate gene expression profiles from human PAs and normal brain samples (GSE42656 and GSE44971). GSE42656 contains 14 PAs and 4 fetal brain controls, whereas GSE44971 has 35 cerebellar PAs and 9 normal cerebellum samples. In both datasets, CCL2 levels were elevated in the PAs relative to their non-neoplastic counterparts (Figure 2A). In addition, since the fusion BRAF rearrangement is a genomic hallmark of optic pathway and cerebellar PAs [3,20], we compared cerebellar PAs to non-neoplastic cerebellar tissue in the GSE44971 dataset, and similarly observed increased CCL2 expression in the tumors (Figure 2B). Moreover, we obtained three short-term primary human PA cell lines established from fresh surgical specimens, and analyzed them for CCL2 mRNA expression. CCL2 mRNA was increased in all 3 PA cell lines relative to normal human astrocytes (Figure 2C), demonstrating that the tumor cells produce CCL2. Third, Ccl2 expression was increased four-fold in independently-isolated f-BRAF-expressing cerebellar NSCs at the protein level (ELISA) (Figure 2D), whereas in cortical NSCs and cerebellar astrocytes, which do not increase their proliferation in response to f-BRAF, did not (Figure 2E). In this regard, previous studies have revealed that f-BRAF expression has both tissue- and cell-type specific effects: Whereas f-BRAF induces mTOR activation and increased cell growth in brainstem and cerebellar NSCs, no increase in mTOR activation or cell proliferation was observed when f-BRAF was expressed in cerebellar astrocytes or cortical NSCs [5,13]. These latter results indicate that the patterning of gliomagenesis is partly dictated by the specific cell of origin (cell type and brain region) [13,14].

Taken together, based on these converging mouse and human data, we sought to focus on Ccl2 as an f-BRAF-regulated chemokine potentially important for recruiting microglia to establish a permissive tumor microenvironment. CCL2 has been previously implicated in tumorigenesis and metastasis in several other solid tumor types [21,22], including high-grade glioma [23,24]. As such, malignant glioma cells elaborate chemoattractants, including CCL2, that promote the directional migration of macrophages and microglia to the developing tumor bed [25–29]. In addition, a correlation between glioma grade and tumor microglia/macrophage content has been demonstrated [30]. However, the role of CCL2 in PA tumorigenesis has not been investigated.

To determine whether Ccl2 is regulated by BRAF-mediated ERK activation, control and f-BRAF-expressing cerebellar NSCs were treated with two different MEK inhibitors, PD0325901 (PD901) and Trametinib, each resulting in decreased ERK activation (Thr202/Tyr204 phosphorylation; Figure 3A). Following either PD901 or Trametinib treatment, Ccl2 mRNA level and protein levels were reduced (Figure 3, B and C), indicating that Ccl2 is regulated by the BRAF-MEK–ERK signaling pathway, consistent with the known function of f-BRAF in MEK/ERK activation [5,11,17–19].

Ccl2 gene expression is transcriptionally controlled at the promoter level by several different transcriptional regulators, including NFKB, C/EBP1, AP-1 and SP-1 [31]. Since NFKB is an important transcription factor for CCL2 regulation in glial cells [32], we sought to determine whether BRAF-MEK–ERK mediates Ccl2 induction through NFKB activation. NFKB activation is partly regulated through IkB phosphorylation, where the inactive cytosolic form of NFKB becomes associated with IkB. Following IkB phosphorylation, the NFKB heterodimer dissociates from IkB and enters the nucleus in its active form. As shown in Figure 3D, IkB phosphorylation (NFKB activation) is increased in f-BRAF-expressing cerebellar NSCs, and MEK inhibition leads to reduced IkB phosphorylation (Ser32) levels. Lastly, to demonstrate that NFKB activation is responsible for f-BRAF-mediated Ccl2 induction, f-BRAF-expressing cerebellar NSCs were treated with TP-CA1, a selective inhibitor of IkB kinase that inhibits NF-kB nuclear localization. f-BRAF-expressing NSCs treated with TP-CA1 showed reduced IkB phosphorylation (Ser32) level and decreased Ccl2 mRNA expression (Figure 3E), thus establishing that f-BRAF regulates Ccl2 expression in a MEK/NFKB-dependent manner. The finding that Ccl2 induction following f-BRAF expression in cerebellar NSCs involves MEK/ERK-dependent IkB kinase and NFKB activation is consistent with previous reports in other cell types (macrophages), where lipopolysaccharide (LPS)-induced NFKB activation is dependent upon ERK activation through the prevention of activate IkB kinase degradation [33]. In addition, MEK/ERK signaling pathway activation is similarly important for TNFa-induced CCL2 expression in human proximal tubular epithelial cells [34].

Since Ccl2 could potentially function in an autocrine manner, as previously reported for CXCL12 and CCL5 [35,36], we sought to determine whether Ccl2 could promote f-BRAF-expressing cerebellar NSC growth. Because Ccl2 can act through its receptors, Ccr2 and Ccr4, to increase NSC growth, we first examined Ccl2 receptor expression, and found that cerebellar NSCs express only the Ccr4 receptor (Figure 4A). Second, we added recombinant mouse Ccl2 (200 ng/ml) to control and f-BRAF-expressing cerebellar NSCs, but observed no significant increase in proliferation (Figure 4B). Third, we assessed the effect of Ccl2 silencing on f-BRAF-expressing NSC
proliferation. shRNA-mediated Ccl2 knockdown using two independently derived shRNA constructs had no effect on f-BRAF-expressing cerebellar NSC proliferation (Figure 4C). Taken together, Ccl2 does not increase f-BRAF-expressing cerebellar NSC growth, suggesting that its primary effect is paracrine, as previously shown for human malignant glioma cell lines [37].

To determine whether CCL2 was responsible for microglia infiltration and glioma-like lesion formation in vivo, f-BRAF-
Figure 3. Increased Ccl2 by f-BRAF is mediated by MEK–ERK–NFκB activation. (A) Increased ERK phosphorylation in f-BRAF-expressing cerebellar NSCs was attenuated following treatment with 10 nM PD0325901 (PD901; top) or 50 nM Trametinib (Tra; bottom). α-tubulin is an internal protein loading control. Veh, Vehicle. (B) Increased Ccl2 mRNA levels in f-BRAF-expressing cerebellar NSCs were reduced to control cell levels following treatment with PD901 (top) or Trametinib (Tra; bottom). (C) Increased Ccl2 protein levels in f-BRAF-expressing cerebellar NSCs were reduced to control levels following PD901 or Trametinib treatment as measured by ELISA. (D) Phospho-IκBα (Ser32) was decreased in f-BRAF-expressing cerebellar NSCs following PD901 or Tra treatment. (E) The increased phospho-IκBα (Ser32) in f-BRAF-expressing cerebellar NSCs was decreased following treatment with 500 nm TPCA-1. Increased Ccl2 mRNA level in f-BRAF-expressing cerebellar NSCs was reduced to control cell levels following TPCA-1 treatment.
expressing cerebellar NSCs were injected into the cerebella of 4-week-old wild type and Ccr2-deficient mice (Ccr2\textsuperscript{RFP}/Ccr2\textsuperscript{RFP} mice). Using this method, low-grade glioma-like form between 3 and 6 months after the injection of \textit{f-BRAF}-expressing cerebellar NSCs. In striking contrast, no tumors form in mice engrafted with wild type (control) cerebellar NSCs. However, in both cases, the injection site exhibited increased glial fibrillary acid protein (GFAP) immunoreactivity, thus enabling the identification of the site of injection in each mouse (Figure 5). Since \textit{f-BRAF}-expressing NSCs express green fluorescent protein (GFP), GFP\textsuperscript{+} cells were only identified at the injection sites of \textit{f-BRAF}-transplanted mice. Consistent with our previous study, at 6 months post-injection, \textit{f-BRAF}-expressing NSC-engrafted wild type mice (n = 5) had increased numbers of proliferating (Ki67\textsuperscript{+}) cells and increased microglia (Iba1\textsuperscript{+} cells) infiltration relative to control NSC-injected wild type mice (n = 5) (Figure 5). In striking contrast, Ccr2-deficient mice bearing \textit{f-BRAF}-expressing NSCs (n = 5) showed reduced numbers of proliferating cells and microglia infiltration relative to wild-type mice. Given the absence of an autocrine effect, these results indicate that Ccl2 establishes a supportive microenvironment for gliomagenesis through Ccr2-mediated microglia recruitment.

The importance of microglia and macrophages to glioma formation, growth and invasion is further underscored by several studies in which pharmacologic or genetic silencing of microglia/macroage function attenuates malignant glioma growth [27,38–40]. Moreover, in \textit{Nf1} mouse models of low-grade glioma,
we have previously shown that microglia are critical for glioma formation and growth [6, 8, 41] through the elaboration of key chemokines [35], specifically Ccl5 [42].

While the mechanism underlying microglia support of KIAA1549: BRAF-associated low-grade glioma growth remains to be elucidated, we have excluded numerous chemokines previously implicated in Nf1 mouse low-grade glioma pathogenesis, including Ccl5 and Cxcl12 (data not shown). Future investigations will be required to identify the responsive microglia-produced growth factors that support sporadic low-grade glioma growth. Nonetheless, in the current study, we establish that Ccl2 is the major chemokine produced by KIAA1549: BRAF-expressing NSCs important for low-grade glioma-like lesion formation in mice. In addition to demonstrating the molecular mechanism responsible for MEK-driven Ccl2 transcription, we demonstrate that Ccr2 is required for both microglia infiltration and low-grade glioma-like lesion formation in vivo. Collectively, these findings reveal how the frequently reported PA genetic alteration, KIAA1549:BRAF creates a permissive low-grade glioma microenvironment in a paracrine fashion through the Ccl2/Ccr2 axis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.11.007.

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