Midkine activation of CD8$^+$ T cells establishes a neuron-immune-cancer axis responsible for low-grade glioma growth

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Brain tumors (gliomas) are heterogeneous cellular ecosystems, where non-neoplastic monocyctic cells have emerged as key regulators of tumor maintenance and progression. However, relative to macrophages/microglia, comparatively less is known about the roles of neurons and T cells in glioma pathobiology. Herein, we leverage genetically engineered mouse models and human biospecimens to define the axis in which neurons, T cells, and microglia interact to govern Neurofibromatosis-1 (NF1) low-grade glioma (LGG) growth. NF1-mutant human and mouse brain neurons elaborate midkine to activate naïve CD8$^+$ T cells to produce Ccl4, which induces microglia to produce a key LGG growth factor (Ccl5) critical for LGG stem cell survival. Importantly, increased CCL5 expression is associated with reduced survival in patients with LGG. The elucidation of the critical intercellular dependencies that constitute the LGG neuroimmune axis provides insights into the role of neurons and immune cells in controlling glioma growth, relevant to future therapeutic targeting.

https://doi.org/10.1038/s41467-020-15770-3

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Numerous studies in malignant solid tumors have highlighted the importance of non-neoplastic stromal cells, particularly cancer-associated fibroblasts, in tumor formation, growth, and progression\(^1,2\). However, in nervous system cancers, fibroblasts are not part of the tumor microenvironment, suggesting that other non-neoplastic cells, including neurons and immune cells (T cells, microglia, and macrophages), could serve this critical function. In this respect, both brain and nerve sheath tumors are characterized by the presence of neurons and infiltrating immune cells\(^3,4\), which could each provide cancer cell support.

These cellular interactions have been more extensively studied in malignant brain tumors (e.g., glioblastoma and diffuse midline glioma), where cancer cells attract resident microglia and peripheral macrophages through the elaboration of chemokines, which in turn produce growth factors that promote tumor growth and progression\(^2\). Similarly, neurons can also regulate malignant brain tumor growth involving several mechanisms, ranging from activity-dependent release of growth factors to tonic release of neuropeptides and neurotransmitters\(^6,7\).

In contrast, these stromal dependencies are likely to be greater in benign tumors with fewer genetic mutations, such as those arising in the setting of cancer predisposition syndromes, like neurofibromatosis type 1 (NF1), in which affected patients develop low-grade peripheral nerve sheath tumors and brain tumors (optic pathway gliomas; OPGs). Whereas peripheral nerve sheath tumors require growth factors from mast cells and macrophages\(^3,8\), NF1-OPGs depend on resident brain monocytes (microglia) for their formation and progression\(^9,10\). In NF1 murine optic gliomas, microglial production of a key growth factor (Ccl5) is both necessary and sufficient for tumor formation and growth\(^11,12\). Importantly, microglial Ccl5 expression requires T lymphocytes, such that glioma formation does not occur in mice lacking functional T cells\(^12\). However, it is currently not known how T cells are recruited to the developing tumor, how they are activated, and how their activation results in microglia Ccl5 production.

In light of the intimate association of these tumors with nerves and the increasing recognition that neurons can provide instructive signals to cancer cells, we sought to dissect the critical tumor-promoting axis involving neurons, immune cells, and low-grade gliomas (LGG) cancer cells using numerous converging cellular and molecular methodologies. Herein, we describe the complex cellular and molecular interactions between neurons, T cells, microglia, and glioma cells that comprise the LGG ecosystem, revealing critical roles for neurons and T cells in glioma formation and maintenance. We demonstrate that human and mouse NF1-mutant neurons produce midkine to activate T cells, which in turn leads to increased T cell Ccl4 secretion and the priming of microglia to elaborate Ccl5 to maintain LGG cell growth. Moreover, we identify each of the responsible intracellular signaling pathways and show that inhibiting integrin-mediated T cell brain entry or function is sufficient to attenuate glioma growth in vivo. The discovery of this co-dependent neuron/T cell/microglia/cancer cell axis provides opportunities for future pediatric LGG therapeutic targeting.

**Results**

**T cell Ccl4 induces microglial Ccl5 production.** Previous studies from our laboratory demonstrated that only activated, but not naïve, T cells produce paracrine factors that induce microglia to produce Ccl5 (Fig. 1a)\(^10\). To identify the paracrine factors induced by T cell activation, we employed a commercially available cytokine array to query the conditioned medium (CM) from activated (anti-CD3/CD28-mediated activation) versus naïve (non-activated) T cells in triplicate. In total, nine cytokines/chemokines (i.e., TNFxα, GM-CSF, Ccl2, Ccl1, Ccl3, Ccl4, Ccl5, IL-1ra, and IL-2) were increased in activated, relative to non-activated, T cell CM (Fig. 1b and Supplementary Fig. 1a). We tested the ability of these cytokines to induce microglial Ccl5 production. After validating the increased expression of these nine cytokines, and the unaltered expression of two additional cytokines (Il10 and Il1f7) (Fig. 1c and Supplementary Fig. 1b) as negative controls, we treated WT microglia with each cytokine at the same concentrations measured in the activated T cell CM, and determined the microglia Ccl5 expression levels by ELISA. Only Ccl4 increased microglial Ccl5 protein expression to the levels seen following activated T cell CM treatment in both adult and neonatal microglia (Fig. 1a, d and Supplementary Fig. 1c, d). Similar results were obtained using different concentrations of these candidate cytokines (Supplementary Fig. 2a–h). While microglia were the major cell type expressing Ccl5 in the murine Nf1 optic gliomas (Supplementary Fig. 1e), activated T cells produced some Ccl5 (Fig. 1a), which could contribute to the Ccl5 induction observed in our experimental paradigm. To exclude T cell Ccl5 from the observed microglial response, activated Ccl5-/- T cells were analyzed. Ccl5-deficient T cell CM elicited a similar increase in microglial Ccl5 secretion, demonstrating that the Ccl5 produced by T cells did not contribute in this setting (Supplementary Fig. 2i).

Using the Immgen database, Ccl4 expression is enriched in several T cell populations, including regulatory T cells (Tregs) and CD8+ T cells (Supplementary Fig. 2j). To determine whether Ccl4 is necessary for T cell CM-induced microglial Ccl5 production, a combination of Ccl4-neutralizing antibodies and Cc4 receptor (Ccr5 and Ccr8) inhibitors were employed: Ccl4-neutralizing antibodies reduced activated T cell-induced microglia Ccl5 production by >60% (Fig. 1e). While both Ccr5 and Ccr8 were expressed by microglia (Fig. 1f), neither inhibiting Ccr5 (MCV treatment) or Ccr8 (AZ058 treatment) alone reduced Ccl5 to the same level as Ccl4-neutralizing antibodies (Fig. 1g). However, the combination of Ccr5 and Ccr8 inhibition (MCV + AZ058) reduced activated T cell-induced microglia Ccl5 production by ~60%, comparable to the effect observed with Ccl4-neutralizing antibodies (Fig. 1e, g). As controls, microglia were exposed to non-activated T cell CM in the presence or absence of Cc4 receptor inhibitor, with no effect on microglia Ccl5 production (Supplementary Fig. 2k). Since Ccl5 inhibits the apoptosis of Nf1-deficient high-grade glioma cells\(^13\), we analyzed the biological consequence of each of these reductions in Ccl5 expression on optic glioma stem cell (o-GSC) survival. Decreasing Ccl5 concentrations from 700 pg ml\(^{-1}\) to 400 or 200 pg ml\(^{-1}\), representing the Ccl5-inhibitory effect from anti-Cc4 and Ccr5/Ccr8 blockers, increased o-GSC apoptosis by 50% or to untreated baseline levels, respectively (Supplementary Fig. 2l), thus establishing a clear biological effect of this Ccl4 axis inhibition on microglial support of LGG stem cell survival.

Ccl3 also increased microglia Ccl5 production, albeit to a far lesser extent (2.5-fold increase vs.18.5-fold increase in response to Cc4; Fig. 1d). However, the combination of Cc3 and Cc4 together did not further increase microglial Ccl5 expression beyond what was seen with Cc4 alone (Supplementary Fig. 2m). Taken together, these results establish Ccl4 as the cytokine responsible for inducing microglia Ccl5 production relevant to glioma growth regulation.

MDK from NF1-mutant neurons induces T cell Ccl4 production. Since our experimental paradigm involved a non-physiologic method of T cell activation, we next sought to identify the factor(s) responsible for NF1-mediated T cell activation in the setting of
For these studies, we leveraged both human-induced pluripotent stem cells (hiPSCs) harboring actual NF1 patient germline NF1 gene mutations, as well as Nf1-mutant mice. Human neurons were generated from isogenic hiPSCs either heterozygous (NF1-het) or homozygous (NF1-null) for two NF1 patient germline NF1 gene mutations [c.2041C>T and c.6576C>T] using established protocols\(^{13}\). In light of previous work demonstrating elevated midkine (MDK) levels in NF1 patient samples, including low-grade peripheral nerve sheath tumors (neurofibromas\(^{14}\)) and skin\(^{15}\), we employed a commercial array containing MDK and other cytokines. Using this assay, NF1-null neurons secreted higher levels of MDK and CSF-2 relative to control neurons (Supplementary Fig. 3a). Quantitative real-time polymerase chain reaction (qRT-PCR) validation revealed increased MDK, but not CSF2, mRNA expression in NF1-null, relative to control, neurons (Supplementary Fig. 3b, c). In contrast, increased CSF-2 protein levels were detected by ELISA in the CM of both NF1-null neurons relative to control neurons, but at much lower levels relative to MDK [CSF-2: 100–300 pg ml\(^{-1}\) (Supplementary Fig. 3d), MDK: 40,000–50,000 pg ml\(^{-1}\) (Fig. 2a)].

Neuron-derived MDK or CSF-2 could influence T cell biology in two ways. First, it could function as a chemoattractant for T cells. However, CD3\(^+\) T cell migration measured using a transwell assay was not increased in response either to MDK or

\[ C_{CM}^{MDK} = 40,000 \text{ pg ml}^{-1} \]

\[ C_{CM}^{CSF-2} = 100 \text{–} 300 \text{ pg ml}^{-1} \]

Neuron-derived MDK or CSF-2 could influence T cell biology in two ways. First, it could function as a chemoattractant for T cells. However, CD3\(^+\) T cell migration measured using a transwell assay was not increased in response either to MDK or CSF-2. The data suggest that MDK may have a specific role in T cell migration, potentially by influencing other chemokines or signaling pathways.

**Figure 2**

- **a**: Graph showing the concentration of CCL5 (pg/ml) in CM from different treatment groups. The x-axis represents different treatments, and the y-axis shows the concentration of CCL5. The graph indicates that the concentration of CCL5 is significantly higher in the NF1-null group compared to the control group.
- **b**: Table listing the fold increase and p-values for various cytokines. The table includes TNF\(\alpha\), GM-CSF, CCL2, CCL1, CCL3, CCL4, CCL5, IL-1ra, and IL-2. The fold increase values range from 1.29 ± 4.5 to 4.0 ± 4.4, with corresponding p-values ranging from 0.046 to 0.415.
- **c**: Graph showing the concentration of TNF\(\alpha\) and GM-CSF in CM from different treatment groups. The x-axis represents different treatments, and the y-axis shows the concentration of TNF\(\alpha\) and GM-CSF. The graph indicates that both TNF\(\alpha\) and GM-CSF concentrations are significantly higher in the NF1-null group compared to the control group.
- **d**: Graph showing the concentration of CCL5 in CM from different treatment groups. The x-axis represents different treatments, and the y-axis shows the concentration of CCL5. The graph indicates that the concentration of CCL5 is significantly higher in the NF1-null group compared to the control group.
- **e**: Graph showing the concentration of CCL5 in CM from different treatment groups. The x-axis represents different treatments, and the y-axis shows the concentration of CCL5. The graph indicates that the concentration of CCL5 is significantly higher in the NF1-null group compared to the control group.
- **f**: Graph showing the concentration of CCL5 in CM from different treatment groups. The x-axis represents different treatments, and the y-axis shows the concentration of CCL5. The graph indicates that the concentration of CCL5 is significantly higher in the NF1-null group compared to the control group.
- **g**: Graph showing the concentration of CCL5 in CM from different treatment groups. The x-axis represents different treatments, and the y-axis shows the concentration of CCL5. The graph indicates that the concentration of CCL5 is significantly higher in the NF1-null group compared to the control group.
Fig. 1 Activated T cells secrete Ccl4 and prime microglia to produce Ccl5. a Activated T cell conditioned medium (act-Tm CM) treatment stimulated WT microglia (MG) to produce a higher level of Ccl5 (ELISA) compared to non-activated T cell-conditioned medium (non-act-Tm CM). Non-activated and activated T cell CM, and CM collected from microglia alone served as controls for these experiments. b WT T cells isolated from the mouse spleen were seeded at the concentration of 2.5 x 10^6 cells ml^-1 in complete PRIM1640 medium followed by 2 days of CD3/CD28 stimulation (activated; act-Tm) or vehicle (PBS) treatment (non-activated; non-act-Tm). CM was collected for chemokine array triplicates. Increased levels of TNF-α, GM-CSF, Ccl2, Ccl3, Ccl4, Ccl5, Il-1ra, and Il-2 expression were observed in activated T cell CM relative to non-activated T cell CM. The fold increases and P values relative to control groups for all three replicates (Supplementary Fig. 1a) are collated in the table. c ELISA assays reveal increased levels of TNFα, GM-CSF, Ccl2, Ccl3, Ccl4, Ccl5, Il-1ra, and Il-2 in the CM of activated, relative to non-activated, T cells. d WT microglia were stimulated with these differentially expressed cytokines (TNF-α (400 pg ml^-1), GM-CSF (1000 pg ml^-1), Ccl2 (80 pg ml^-1), Ccl3 (500 pg ml^-1), Ccl4 (8000 pg ml^-1), Il-1ra (80 pg ml^-1), and Il-2 (6000 pg ml^-1)) for 24 h at the concentrations detected in the activated T cell CM. Ccl5 production by microglia was increased following Ccl4 (6000 pg ml^-1) treatment. Veh: vehicle. e Ccl5 ELISA revealed that activated T cell CM induction of microglial Ccl5 production was reduced following treatment with increasing concentrations of Ccl4 neutralizing antibody. f Microglial Ccr5 and Ccr8 expression was validated using spleen as a positive control, g increasing concentrations of maraviroc (MCV, Ccr5 receptor inhibitor) and AZ084 (Ccr8 receptor inhibitor) reduced T cell induction of microglial Ccl5 expression. The combination of MCV and AZ084 exhibited the greatest inhibition of microglial Ccl5 expression. All data are presented as the mean ± SEM. a This representative experiment was conducted with n = 3 independent biological samples, and was replicated two additional times with similar results. b n = 3 independent biological samples were examined over three independent experiments, as illustrated in Fig. 5a. c and d Bar graphs represent the means ± SEM of n = 3 independent biological samples. e This representative experiment was conducted with 0 mg ml^-1 anti-Ccl4, n = 6, 1, 2, 2.5 mg ml^-1 anti-Ccl4, n = 3, independent biological samples, and was replicated two additional times with similar results. f Bar graphs represent the means ± SEM of n = 4 independent biological samples. g This representative experiment was conducted with (from left to right) n = 7, n = 6, n = 6, n = 4, n = 5, n = 4, and n = 5 independent biological samples, and was replicated two additional times with similar results. a, d, e, g One-way ANOVA with Bonferroni post-test correction; b, c, f Two-tailed Student’s t-test. Exact P values are indicated within each panel; N.S.: not significant. From left to right in each panel: a P < 0.001, c P < 0.001, d N.S., N.S., N.S., N.S., P = 0.035, P < 0.001, N.S., N.S., e P = 0.025, P < 0.001, P < 0.001; f N.S.; g P = 0.012, P < 0.001, P < 0.001, P < 0.001.
Moreover, MDK-induced T cell Ccl4 production was reduced following treatment with the FK506 and cyclosporin (calcineurin inhibitors24; Fig. 3g). FK506 treatment also inhibited hiPSC-derived neuron CM induction of T cell Ccl4 production (Fig. 3h). Collectively, our findings demonstrate that MDK binds to Lrp1 on T cells and induces Ccl4 expression through calcineurin-mediated NFAT1 nuclear translocation.

**CD8**+ T cell brain entry is required for optic glioma growth. While the above studies revealed important roles for neurons and T cells in LGG stroma-tumor circuitry, they do not establish whether T cells are necessary for optic glioma growth. To demonstrate a requirement for T cells and directed T cell infiltration in murine Nf1 optic glioma growth, we performed two preclinical proof-of-principle experiments using neutralizing antibodies. First, Nf1 OPG mice were treated with anti-CD3 antibodies to deplete T cells from 6 weeks of age until 3 months of age, verified by flow cytometry of splenic cells (IgG group, 55% CD3+ cells; anti-CD3 group, 7.61%; Fig. 4a). Relative to isotype antibody (IgG) treatments, the OPGs in these mice had reduced numbers of CD3+ T cells and attenuated tumor proliferation (Fig. 4b). Second, since T cells are rarely found in the normal brain parenchyma, but are detected in NF1-associated mouse and human LGGs12,27, we examined the meningeal space in control (CTL, Nf1fl/flox/flox) and Nf1 optic glioma (OPG, Nf1flox/mut; GFAP-Cre) mice as a potential point of entry28. Similar to the optic nerves27, more CD3+ T cells were observed in the meningeal space of OPG mice relative to controls (Fig. 4c), suggesting that T cells in the setting of optic gliomas are most likely recruited...
Fig. 2 NF1-mutant neurons express MDK, which activates T cells to produce Ccl4. a Isogenic hiPSC-induced neurons with heterozygous NF1 patient NF gene mutations (2041C>T and 6576C>T) produced higher levels of mIlkine in the neuron conditioned medium (N-CM) compared to WT (CTL) hiPSC-induced neurons. b MDK gene expression was higher in the optic nerves of NF1+/− relative to WT mice. c Increased MDK expression was observed in optic glioma (OPG)-containing relative to control (CTL) optic nerves. d No change in T cell migration was observed in response to various MDK concentrations. e MDK (50 ng ml−1) stimulation for 48 h increased T cell Ccl4 production. f CM from isogenic hiPSC-induced neurons with NF1 patient NF gene mutations (c.2041C>T-N-CM and c.6576C>T-N-CM) exhibited a stronger T cell Ccl4 induction compared to CM from control hiPSC-induced neurons (CTL-N-CM). Anti-MDK neutralizing antibodies reduced T cell Ccl4 production in response to hiPSC-induced neuron CM stimulation. g MDK-activated (50 ng ml−1) T cell CM (mid-treated Tm) increased microglial Ccl5 production relative to non-activated T cell CM (non-act-Tm). h Immunohistochemistry revealed an increased percentage of MDK-immune reactive cells in the retinal ganglion cell layer of OPG-bearing and independent biological samples. Scale bars, 40 μm. Arrows denote representative immunopositive cells. i MDK gene expression was examined in NF1 pilocytic astrocytomas (NF1-PAs, n = 9), non-NF1-PAs (n = 9), and non-neoplastic brain tissues (n = 4). Increased MDK expression was detected in NF1-PAs compared to normal brain tissue and non-NF1-PAs. Arrows denote representative immunopositive cells. All data are presented as the mean ± SEM. a–c: These representative experiments were conducted with a CTL, n = 3, 2041C>T n = 4, 6576C>T, n = 3; b WT, n = 7, Nf1−/−, n = 6; c CTL, n = 5, OPG, n = 4, independent biological samples, and were replicated two additional times with similar results. d and e Bar graphs represent the means ± SEM of d n = 5, e n = 3, independent biological samples. f This representative experiment was conducted with (from left to right) n = 3, n = 3, n = 4, n = 3, n = 5, and n = 3 independent biological samples, and were replicated two additional times with similar results. g Bar graphs represent the means ± SEM of non-activated Tm, n = 3; MDK-treated Tm, n = 4 independent biological samples. h Bar graphs represent the means ± SEM of WT n = 9, NF1−/−, n = 7, CTL, n = 5, OPG, n = 7, independent biological samples. i Bar graphs represent the means ± SEM of CTL, n = 4, non-NF1, PA, n = 9, Nf1−/−, n = 9, independent biological samples. a, b, f, i One-way ANOVA with Bonferroni post-test correction, b, c, h Two-tailed Student’s t-test. Exact P values are indicated within each panel; N.S., not significant. From left to right in each panel: a all P < 0.001; b P = 0.006; c P = 0.021; d all N.S.; f top P = 0.012, middle P = 0.023, P = 0.030, bottom P = 0.045, P = 0.039; i CTL:Nf1 PA P = 0.024, non-NF1 PA:Nf1-PA P = 0.007; h all P < 0.001.

from this location into the evolving tumor. Because T cell migration from the meningeal space is mediated by integrins, such as the very late antigen 4 (VLA4)29, NF1 OPG mice were treated with anti-VLA4 antibodies in a parallel experiment. Consistent with a dependency on integrin for T cell parenchymal invasion, anti-VLA4 treatment inhibited CD3+ T cell migration from the meningeal space is mediated by integrins, such as the very late antigen 4 (VLA4)29, rather than CD4+ T cell infiltration, as observed following MDK-treated CD3+ T cells (Fig. 4b). Interestingly, neither treatment affected microglia content (Iba1+ cells, Fig. 4b).

Next, we sought to determine which T cell population predominated in these tumors using CD4, CD8, and Foxp3 antibodies. Whereas no Foxp3+ cells were detected in the OPGs (Supplementary Fig. 5a), the majority of the T cells in the tumors were CD8+ T cells, rather than CD4+ T cells (Fig. 5a). The cellular identity of these CD8+ T cells in the mouse NF1 OPGs were verified as CD3+ T cells by double labeling immunohistochemistry (Fig. 5b). A similar pattern was also observed in the meninges of these mice, where the vast majority of the T cells were CD8+, with a smaller infiltration of CD4+ cells (Fig. 5c). Consistent with these findings in murine OPGs, CD8+ T cells were also detected in human NF1-PAs (N = 4; Supplementary Fig. 5b).

To establish a requirement for CD8+ T cells in NF1 OPG growth, we employed anti-CD8 antibody treatment to deplete CD8+ T cells using a protocol similar to that described for the anti-CD3 treatments. CD8+ T cell depletion was validated in splenocytes by antibody exposure, the OPGs in treated mice had reduced no change in microglia content (Fig. 6a). Consistent with a key role for this population of T lymphocytes in LGG pathobiology and similar to CD3+ T cells, CD8+ T cells produced Ccl4 in response to MDK (Supplementary Fig. 5d), which was inhibited by MDK-neutralizing antibodies (Supplementary Fig. 5d). Additionally, microglia exposed to MDK-treated CD8+ T cell CM exhibited similar levels of microglial Ccl5 (Supplementary Fig. 5e) as observed following MDK-treated CD3+ T cell CM exposure (Fig. 2g). Consistent with the T cell Ccl4 regulation of microglial Ccl5 expression, anti-CD8 antibody treatment of NF1 OPG mice reduced the percent of Ccl4+ and Ccl5+ cells in the murine LGGs (Fig. 6a).

 Lastly, to explore the possible clinical relevance of CD8+ T cell infiltration, we analyzed CD8 and CD4 RNA expression in datasets with LGG [Brain Lower Grade Glioma (TCGA, Provisional, overlapping patients excluded) and Brain Lower Grade Glioma (TCGA, PanCancer Atlas) samples]30. In both LGG datasets, shorter overall survival correlated with increased CD8, but not CD4, expression (Fig. 6b, c). Similarly, increased CD8 expression also correlated with reduced progression-free survival in the one dataset (TCGA, Provisional) with sufficient numbers of samples for analysis (Supplementary Fig. 5f). Taken together, these findings demonstrate that CD8+ T cell entry into the brain is required for NF1 OPG growth, which is also associated with poor clinical outcome in patients with LGG.

T cell-induced microglial Ccl5 production inhibits apoptosis. Additionally, we sought to elucidate the mechanisms responsible for microglia Ccl5 production and LGG cancer cell growth. Initial experiments focused on defining the monocyte population (macrophages versus microglia) and the effect of a germline NF1 gene mutation on the involved monocyte population. First, while the monocytes that populate the murine NF1 OPGs are CD11b+/CD45low/Cx3cr1+ cells (microglia)39, rather than peripheral CD11b+/CD45high/Cx3cr1+ macrophages, we nevertheless examined the responses of WT splenocytes (macrophages) to activated T cell CM (Supplementary Fig. 6a). In striking contrast to the results obtained with microglia, no significant induction of Ccl5 in splenic monocytes was observed, supporting a T cell-microglia, rather than T cell-macrophage, circuit in the maintenance of NF1 OPGs. Second, because the stromal (T cells and microglia) cells in both murine and human NF1-OPG are heterogeneous for a germline NF1 gene mutation, we also examined the ability of NF1+/− T cells and microglia to interact and produce Ccl5. Similar induction of microglial Ccl5 was observed using activated NF1+/− T cell CM or NF1+/− microglia (Supplementary Fig. 6b, c). For these reasons, we explored the regulation of Ccl5 using WT T cells and microglia.

Since NFkB has been previously implicated in Ccl5 transcriptional regulation26, we examined NFkB inhibitor (Iκbα) phosphorylation in microglia following exposure to activated T cell CM. Increased Iκbα phosphorylation, indicating higher levels of NFkB activity, was observed following activated T cell CM treatment, which was abrogated using an NFkB inhibitor (Caffeic acid phenethyl ester, CAPE) (Fig. 7a). Importantly, increased
Fig. 3 MDK activates T cells to produce Ccl4 through Lrp1/calcineurin/NFAT1 signaling. 
a T cells expressed only two (Lrp1 and Lrp6) of the putative MDK receptors (protein-tyrosine phosphatase ζ (Ptprz1), neuroglycan-C (Cspg5), low density-lipoprotein receptor-related protein-1 (Lrp1), low density-lipoprotein receptor-related protein-6 (Lrp6) and anaplastic lymphoma kinase (Alk)) by quantitative RT-PCR. Normal mouse cortex was used as an internal positive control. 
b Lrp1 blocking antibodies (30 µg ml⁻¹) reduced MDK-induced Ccl4 production in T cells. The 2041C>T neuron conditioned media (N-CM)-mediated Ccl4 production in T cells was attenuated following exposure to Lrp1 receptor blocking antibodies. 
d Immunoblotting revealed increased NFAT1 nuclear localization in T cells following MDK treatment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATA-binding protein (TBP) served as loading controls for the cytoplasm and nuclear fractions, respectively. 
e Decreased levels of phosphorylated-NFAT1 (p-NFAT) were observed after MDK stimulation of T cells. Lrp1 blocking antibodies (anti-Lrp1, 30 µg ml⁻¹) increased NFAT1 phosphorylation and impaired NFAT1 nuclear localization in MDK-stimulated T cells. The calcineurin inhibitor FK506 (10 µM) inhibited MDK-induced NFAT1 nuclear localization. 
g Calcineurin inhibitors, cyclosporin (100 nM) and FK506 (10 µM), inhibited MDK-induced Ccl4 production in T cells. h FK506 (10 µM) reduced 2041C>T neuron conditioned media (N-CM)-induced Ccl4 production in T cells. All data are presented as the mean ± SEM. 
a Bar graphs represent the means ± SEM of n = 3 independent biological samples. 

One-way ANOVA with Bonferroni post-test correction. 

Two-tailed Student's t-test. Exact P values are indicated within each panel; N.S.; not significant. From left to right in each panel: b P = 0.005, P = 0.007; c N.S., P = 0.007; g P < 0.001, N.S., N.S.; h P < 0.001. 

d–f These are representative images of n = 3 independent biological samples examined over three independent experiments with similar results. Molecular weight markers are denoted at the left side of each blot.
microglial Ccl5 production in response to activated T cell CM exposure was blocked by NFκB inhibition (CAPE; Fig. 7b).

Based on prior studies demonstrating that Ccl5 is necessary for murine NFI optic gliomas, we next sought to determine whether Ccl5 increases the survival of cancer stem cells from murine NFI optic gliomas (optic glioma stem cells; o-GSCs). We focused on these cells, since they are capable of generating glioma-like lesions in mice following transplantation, and no suitable primary human NFI-LGG cell lines are currently available for analysis. Whereas Ccl5 treatment in the growth phase (non-confluent) did not change the percentage of proliferating (Ki67\(^+\)) or-GSCs (Fig. 7c), Ccl5 reduced the apoptosis of confluent o-GSC cultures (TUNEL staining; Fig. 7d).

To identify the signaling pathway underlying Ccl5 control of o-GSC apoptosis, we performed a survey of known NFI protein (neurofibromin) downstream effectors. While no change in ERK activity was observed following Ccl5 treatment (Supplementary Fig. 6d), Akt and CREB phosphorylation was increased (Fig. 7e). Since Akt activates the CREB pathway through GSK3β in hepatocytes\(^32\), we measured GSK3β activity. Consistent with Akt/GSK3β/CREB hyperactivation as the responsible pathway underlying Ccl5-mediated o-GSC survival, Akt inhibition (MK2206) following Ccl5 treatment attenuated Akt, GSK3β, and CREB activation (using phospho-specific antibodies), as well as increased o-GSC apoptosis (TUNEL) (Fig. 7e, f).

We have previously shown that CD44 is the CCL5 receptor on NFI-deficient high-grade glioma cells\(^33\). To determine whether Ccl5 functions through this receptor, we first analyzed o-GSC CD44 mRNA and protein expression (Fig. 7g,h). Second, following CD44 knockdown in o-GSCs, Ccl5 no longer inhibited o-GSC death (Fig. 7i–j). As such, Ccl5 operates to increase low-grade and high-grade glioma survival through CD44 binding and downstream signaling.

Since Ccl5 mediates o-GSC survival via the AKT/GSK3β/CREB pathway, we examined the percentage of p-AKT\(^\text{Ser473}\)-immunopositive cells and p-CREB\(^\text{Ser133}\)-immunopositive cells following anti-CD8 treatment in NFI optic glioma mice. Consistent with T cell Ccl4 induction of microglial Ccl5-mediated optic glioma growth, we observed a reduction in the percentage of p-AKT\(^\text{Ser473}\)-immunopositive cells and p-CREB\(^\text{Ser133}\)-immunopositive cells in the optic gliomas of anti-CD8 antibody-treated mice (Fig. 8a, b).

Finally, in keeping with a key role for Ccl5 and CD44 in LGG, high CCL5 or CD44 expression also correlated with reduced overall survival in the two available TCGA datasets with LGGs.
In a similar regard, we show that CD8+ T cells were detected in murine Nf1 OPG or control optic nerves. Black arrows denote representative immunopositive cells. Scale bars, 20 μm. 

In addition to directly mediate tumor growth, neurons can also contribute to tumorigenesis by modulating immune cell function. In the brain, neurons produce membrane-bound and soluble factors (e.g., CD22^{40}, CD200^{41}, ICAM-5^{42}, semaphorin-3A^{43}, and CX3CL1^{44}) that each can modulate microglia properties. These tightly regulated interactions are not only essential for normal brain development, during which neuronal complement (C1q) expression triggers microglia-mediated synaptic pruning, but can be subverted to drive nervous system disease progression in the setting of peripheral nerve injury^{45} and neurodegenerative disorders^{46}. Similarly, neurons produce TGF-β to increase CD4+ T cell proliferation^{47}, as well as Fas ligand to promote T cell apoptosis^{48}.

Our finding that NFI-mutant mouse and human neurons increase their expression of MDK, which operates to activate CD4+ T cell proliferation^{49}, as well as Fas ligand to promote T cell apoptosis^{50}. While MDK activation of T cells is the operative mechanism underlying NFI-LGG pathogenesis, other etiologies are likely responsible for non-NFI (sporadic) LGG initiation and growth. As such, elevated MDK expression was not observed in non-NFI PA samples relative to normal brain tissue (Fig. 2i), and MDK expression is not correlated with LGG patient survival using available TCGA data (Supplementary Fig. 6f).

Neuronal activity has been shown to drive high-grade glioma growth through the shedding of neuroligin-3, a protein normally involved in neuron–neuron communication^{51}. Third, nerve innervation is a poor prognostic marker in many cancers outside of the nervous system (pancreas and prostate cancer)^{52,53} such that neuron-secreted factors promote tumor initiation, growth, and metastasis^{54,55}. For example, autonomic nerve fibers positively mediate prostate cancer initiation and progression through adrenergic and cholinergic signaling pathways^{56}. In addition, neural progenitor cells (NPCs) can migrate from the brain to prostate tumors, and drive tumor proliferation and metastasis^{57}. In addition to directly mediate tumor growth, neurons can also contribute to tumorigenesis by modulating immune cell function. In the brain, neurons produce membrane-bound and soluble factors (e.g., CD22^{40}, CD200^{41}, ICAM-5^{42}, semaphorin-3A^{43}, and CX3CL1^{44}) that each can modulate microglia properties. These tightly regulated interactions are not only essential for normal brain development, during which neuronal complement (C1q) expression triggers microglia-mediated synaptic pruning, but can be subverted to drive nervous system disease progression in the setting of peripheral nerve injury^{45} and neurodegenerative disorders^{46}. Similarly, neurons produce TGF-β to increase CD4+ T cell proliferation^{47}, as well as Fas ligand to promote T cell apoptosis^{48}.

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T cells normally provide a level of immune surveillance by trafficking from the cerebrospinal fluid through the brain, and draining back into the periphery via lymphatic vessels in the meningeal spaces^{52}. Although not present in large numbers in the healthy brain, the amount of infiltrating T cells is increased in numerous different histologic brain tumor subtypes, each of which displays a unique pattern of immune cell infiltration and
transcriptional signatures. However, the contribution of CD4+ and CD8+ T cells to these tumors remains unclear. For example, pediatric LGG exhibit higher levels of T cell infiltration relative to their malignant counterparts, while CD4+ and Foxp3+ T cell glioma content correlated with increased tumor progression. Similarly, infiltrating T cells correlated with poor patient survival in one study, whereas another demonstrated that CD8+ T cell content is associated with improved patient survival.

Herein, we found that the majority of T cells in these low-grade murine gliomas are CD8+ lymphocytes, such that their depletion attenuated OPG growth in vivo. While these T cells are typically considered cytotoxic in nature, it is possible that they represent a population of exhausted T cells, which could be converted to tumoricidal. Future experiments will be necessary to define their unique functional properties in this particular neoplastic setting. Similarly, whereas VLA-4 is required for CD8+ T cell infiltration into Nf1 optic gliomas, as revealed by the anti-VLA4 antibody experiments, differential VLA-4 expression in CD4+ versus CD8+ T cells does not account for the preferential CD8+ T cell infiltration in Nf1 murine optic gliomas and human NFI54 gliomas (Supplementary Fig. 6g, h). However, since T cells are recruited into murine Nf1 optic gliomas by chemokines (Ccl2 and Ccl12) released by o-GSCs, it is possible that differences in T cell population recruitment may result from differential responses to these chemokines. Future studies are focused on mechanistically defining how CD8+ T cells are selectively recruited into these LGGs.

As systemic immune regulators, T cells have the capability to educate macrophages/microglia in both health and disease. In other brain diseases, infiltrating T cells are in close vicinity to influence microglia/macroglia. In addition, T cells suppress microglia activation in the phase of inflammatory insults, as well as produce cytokines (e.g., IL-4) that can alter microglia function. Similarly, in many types of cancer, T cells interact with tumor-associated microglia/macrophages (TAMs) through the secretion of factors, like IL-4 and IL-13 and alter tumor growth or invasion. This connection is underscored by previous work from our laboratory in which microglia from athymic (nu/nu)
mice exhibit reduced phagocytosis and reduced Ccl5 expression. Consistent with T cell-mediated microglia education, incubating nu/nu mouse brain microglia with activated wild-type (WT) T cells or their CM resulted in restoration of normal Ccl5 levels. The ability of T cells to communicate with microglia to change their physiologic state and functional capabilities provides another mechanism to control brain homeostasis and influence CNS pathobiology. In light of the patient survival curves stratified by CD8 and CCL5 tumor expression (Figs. 6b, 8c, d), these T cell–microglia interactions may be particularly relevant to LGGs, where MDK/CCL4-independent mechanisms involving CD8+ T cells and microglia result in CCL5 support of tumor growth. Additional experiments using other LGG models will be required to extend these findings beyond NF1.

In summary, the neuron–immune–cancer axis elucidated in this study highlights the tightly choreographed interactions between neoplastic cells and their non-neoplastic neighbors (neurons, T cells, and microglia; Fig. 6h). Similar to other contexts in which numerous cell types (microglia, reactive astrocytes, and oligodendrocyte precursor cells) interact to impact on brain function (methotrexate-induced cognitive impairment), the findings in this report expand the concept of the tumor microenvironment and the complex relationships between participating cell types, including neurons, but also offer opportunities to target the tumor ecosystem.

**Methods**

**Mice.** All mice, including Nf1<sup>+/−</sup> mice (neomycin sequence insertion within exon 31 of the murine Nf1 gene), Nf1<sup>fl/fl</sup>, GFAP-Cre (OPG) mice (Nf1<sup>−/−</sup> mice with somatic Nf1 gene inactivation in neuroglial progenitors at E15.5) and Nf1<sup>lox/lox</sup> mice (controls),<sup>30,37</sup> were maintained on a strict C57BL/6 background and used in accordance with an approved Animal Studies Committee protocol at Washington University. Cdl5<sup>−/−</sup> mice were purchased from the Jackson Laboratory (JAX; 005090). Mice were housed in barrier facilities with controlled light-dark cycles (12:12 h) and ad libitum access to food and water.

**Anti-VLA4, anti-CD3, and anti-CD8 treatments.** Six-week-old OPG mice (n = 10/cohort) were injected with 150 μg of anti-VLA4 (IgG2b), anti-CD3 (IgG2b), anti-CD8 (IgG2b), or rat IgG (isotype control, Bio X Cell) intraperitoneally every other day for 6 weeks. The brains and optic nerves were harvested when the mice reached 3 months of age, and the Iba1, Ki67, and CD3-positive cells analyzed by counting the number of cells showing a positive signal after immunohistochemistry.<sup>37</sup>

**Culture of mouse optic glioma stem cells, human iPSCs, and neurons.** Optic nerves/chiasms were dissected from 3-month-old Nf1<sup>−/−</sup> OPG mice and used to generate o-GSCs. Single cell suspensions were obtained by digesting the optic nerve/chiasm in Trypsin Digest Medium, followed by maintenance in neural stem cell (NSC) medium (61% DMEM low glucose medium, 35% neurobasal medium, 4% EGF; 1% BSA) and human iPSCs, followed by differentiation to mature neurons.
Fig. 8 T cell-induced Ccl5/CD44-mediated cell survival underlies Nf1 optic glioma growth. a, b Immunohistochemistry revealed that anti-CD8 antibody treatment reduced the percentage of p-AKT\textsuperscript{Ser473}-expressing and p-CREB\textsuperscript{Ser133}-expressing cells in mouse Nf1 optic glioma specimens. Black arrows denote representative immunopositive cells. Scale bars, 20 \( \mu \)m. Bar graphs represent the means ± SEM of \( n = 4 \) independent biological samples. Two-tailed Students-\( t \)-test. Exact \( P \) values are indicated within each panel; a \( P < 0.001 \); b \( P = 0.002 \). c, d Kaplan–Meier survival curves (Brain Lower Grade Glioma TCGA Provisional [2 left panels; c \( P = 1.29 \times 10^{-6} \), d \( P = 2.06 \times 10^{-3} \]) and TCGA PanCancer Atlas [2 right panels; c \( P = 4.69 \times 10^{-13} \), d \( P = 9.86 \times 10^{-3} \]) datasets) demonstrate that non-overlapping patients with LGG who harbor high CCL5 expression or CD44 expression have reduced survival time. e Schematic representation of the neuron-immune-cancer axis in NF1-LGG. Meningeal T cells infiltrate into the optic glioma in an integrin (VLA-4)-dependent manner, and are activated by MDK produced by Nf1-mutant retinal ganglion cells (neurons) through a RAS-dependent mechanism. This neuron-mediated T cell activation increases CD8\(^+\) T cell Ccl4 production through increased Lrp1/calcineurin signaling, and results in increased NF\( \kappa \)B-dependent microglial Ccl5 expression, culminating in increased glioma growth through Akt/GSK3\( \beta \)/CREB pathway-mediated suppression of cancer (glioma) stem cell apoptosis and increased tumor growth.
2 mM l-glutamine, 1% penicillin/streptomycin; P/S) supplemented with Glutamax (200 mM), EGF (20 ng ml\(^{-1}\)), FGF (20 ng ml\(^{-1}\)), 1% N\(_2\) and 2% B27.

Isogenic human or humanized NRCIC-T or c.6137T>A NF1 patient-specific NFI germline mutations were engineered using CrisPR/Cas9 technology by the Washington University Genome Engineering and iPSC Core Facility (GEiC). NPCs were differentiated by passaging iPSOs onto PLO/ Laminin (Millipore Sigma)-coated plates using ReLeSR (STEMCELL Technologies), followed by seeding at 200,000 cells cm\(^{-2}\) in NPC induction medium [50% DMEM F12 (Gibco), 50% Neurobasal medium (Gibco), supplemented with N2, B27 (Fisher), 2 mM Glutamax (Gibco), 10 mM l-glutathione, 54 mM KCl, 2.6 mM NaHCO\(_3\), 1.3 mM NaH\(_2\)PO\(_4\), 2.7 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.5 mM EDTA, 25 mM glucose, and 1 mM L-glutamine, 1% P/S, and 6% horse serum].

Western blotting. Nuclear protein extracts were obtained using RIPA and protein concentration was determined using the Pierce BCA protein assay kit. Up to 50 µg of protein per extract were separated in SDS–polyacrylamide gels by electrophoresis. After protein transfer onto PVDF membrane, the membranes were incubated with the indicated antibodies. Antibody binding was detected after incubation with a secondary antibody coupled to IRDye. The blots were analyzed using Image Studio Live Ver 5.2 software. Images of the original immunoblots can be found in Supplementary Figs. 8 and 9.

RNA extraction and real-time qRT-PCR. RNA was isolated from cells using the RNaseasy kit (QIAGEN), while RNA was isolated from mouse optic nerves using the Trizol reagent (Life Technologies). Real-time qRT-PCR was performed using the specific primers listed in Supplementary Table 2. ΔΔCT values were calculated using H3Fla as an internal control.

Survival curves. Kaplan–Meier survival curves were obtained from the MSKCC computational biology cancer genomics portal (http://www.cbioportal.org).\(^{35,48}\) These contain two annotated TCGA datasets (Brain Lower Grade Glioma, TCGA, Provisional) and Brain Lower Grade Gliaoma (TCGA, PanCancer Atlas). Statistical significance was set at \(P<0.05\).

Lovastatin treatment. 100,000 human iPSC-derived NPCs were plated on PLO/laminin-coated six-well plates, allowed to differentiate into neurons in neuron maturation media for 6 weeks, and were then treated with varying concentrations of Lovastatin for 24 h. After an additional 12 h, the conditioned media was collected for midkine ELISA measurements.

Primary neonatal microglia isolation and culture. Mouse pups (postnatal day 0–4; PN0–4) were euthanized, and their brains (without the cerebellum and olfactory bulb) were collected and stripped of the overlying meninges. The brains were then washed twice with ice-cold wash buffer and incubated with 3 ml of 0.25% collagenase for 30 min at 37 °C for inactivating trypsin in 4 ml of complete DEMEM culture media (with 10% FBS). The cells were then treated with DNase, resuspended in 10 ml of DEMEM complete media, passed through a 70 µm
strainer, and seeded in flasks. The culture media was changed on day 3, and cells were fed with an additional 3 ml of fresh complete DMEM on day 7. Between days 8 and 14, microglia were isolated by shaking the flasks for 20–25 min at room temperature on a shaker at 250 rpm.

**Splenic monocyte isolation.** For monocyte isolation, spleen single cell suspensions were incubated with CD11b-specific magnetic beads, and captured using a magnetic separator. 5 x 10^5 microglia/monocytes were grown in T cell CM for 24 h

**Statistical analyses.** Data analyses were performed using GraphPad Prism. Unpaired two-tailed Student’s t-tests were used to determine differences between two groups. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons test. Statistical significance was set at P < 0.05. All data are presented as mean values ± SEM. All experiments have been replicated at least three independent times with similar results.

**Data availability** The data are available within the Article, Supplementary Information and available from the authors upon request. The source data for Figs. 1–8 and Supplementary Figs. 1–9 are provided as a Source Data file. Additional data were extracted from the Memorial Sloan Kettering Cancer Center cBioPortal for Cancer Genomics (https://www.cbioportal.org) and the Immunological Genome Project (www.immgen.org).

Received: 1 October 2019; Accepted: 26 March 2020; Published online: 01 May 2020

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**Acknowledgements**

This work was partially funded by a grant from National Institute of Neurological Disorders and Stroke (1-R35-NS07211-01) to D.H.G., while Y.P. was supported by a McDonnell Center for Cellular and Molecular Neuroscience fellowship and C.A. was supported by an R50 Research Specialist Award (1-R50-CA233164-01). The GeiC Center at WUSM engineered the hiPSCs and is subsidized by NCI Cancer Center Support Grant #P30-CA091842.

**Author contributions**

Conceptualization, D.H.G., X.G., and Y.P.; Methodology, D.H.G. S.S., and X.G.; Writing—review & editing, D.H.G.; Investigation, X.G. performed most of the experiments in this report; S.S. performed the antibody treatments and meninges analyses, M.X. performed some of the T cell–microglia interaction experiments; C.A. performed the hiPSC neuronal assays, midkine ELISA, and qRT-PCR experiments and assembled the figures; O.C. performed the statistical analysis; Resources, D.H.G. and S.D.; Supervision, D.H.G.; Funding acquisition, D.H.G.

**Competing interests**

The authors declare no competing interests.

**Additional information**

*Supplementary information* is available for this paper at [https://doi.org/10.1038/s41467-020-15770-3](https://doi.org/10.1038/s41467-020-15770-3).

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**Peer review information** *Nature Communications* thanks Atique Ahmed, Michael Platten, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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