Athymic mice reveal a requirement for T-cell–microglia interactions in establishing a microenvironment supportive of Nf1 low-grade glioma growth

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Pediatric low-grade gliomas (LGGs) frequently do not engraft in immunocompromised mice, limiting their use as an experimental platform. In contrast, murine Neurofibromatosis-1 (Nf1) optic LGG stem cells (o-GSCs) form glioma-like lesions in wild-type, but not athymic, mice following transplantation. Here, we show that the inability of athymic mice to support o-GSC engraftment results from impaired microglia/macrophage function, including reduced expression of Ccr2 and Ccl5, both of which are required for o-GSC engraftment and Nf1 optic glioma growth. Impaired Ccr2 and Ccl5 expression in athymic microglia/macrophages was restored by T-cell exposure, establishing T-cell–microglia/macrophage interactions as critical stromal determinants that support Nf1 LGG growth.

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Immunocompromised mice (e.g., athymic Foxn1−/− mice) are common tools for human tumor xenograft studies, including high-grade gliomas [Morton and Houghton 2007; Venkatesh et al. 2017]. While malignant glioma stem cells readily form tumors in athymic mice, no successful ectopic xenografts have been reported for low-grade glioma (LGG) cells [Raabe et al. 2011]. One of the critical determinants of LGG formation and growth is a permissive microenvironment composed of nonneoplastic cells, including immune system cells. This dependency on immune system-like cells is highlighted by the high percentage (35%–50%) of Iba1+ microglia/macrophages [Simmons et al. 2011] as well as increased numbers of T cells in these tumors [Yang et al. 2011; Griesinger et al. 2013].

Since relative immunodeficiency is required to accept human cells for rodent xenografting studies and since no validated human LGG lines are currently available, genetically engineered mouse (GEM) models of pediatric LGGs have served as experimental platforms to define the role of immune system-like cells in LGG pathogenesis. To this end, Neurofibromatosis-1 (Nf1) GEM strains have been used to define the conditions critical for optic gliomas: grade I pilocytic astrocytomas [PAs] arising in the Nf1 brain tumor predisposition syndrome [Bajenaru et al. 2003; Zhu et al. 2005]. Unlike grade II LGGs, which can progress to high-grade gliomas, Nf1-associated pediatric LGGs do not transform into malignant gliomas [Hoyt and Baghdassarian 1969] and rarely result in reduced life expectancy.

Consistent with a critical role for immune system cells [monocytes] in Nf1 optic glioma formation and maintenance, genetic Cx3cr1 reduction delays tumor formation [Pong et al. 2013], and genetic elimination of CD11b+ microglia/macrophages [Simmons et al. 2011] or pharmacological microglial inhibition [Daginakatte and Gutmann 2007; Daginakatte et al. 2008] reduces optic glioma proliferation. Moreover, murine Nf1 optic glioma-associated microglia express the Ccl5 chemokine such that neutralizing Ccl5 antibodies attenuates optic glioma growth in vivo [Solga et al. 2015].

Within these murine Nf1 optic gliomas are cancer stem cells, termed optic glioma stem cells (o-GSCs), which are capable of self-renewal, multilineage differentiation, and the generation of glioma-like lesions following transplantation into the brain stems of naïve immunocompetent wild-type mice [Chen et al. 2015]. Interestingly, o-GSCs do not form tumors in immunocompromised athymic [Foxn1−/−] mice, suggesting that these T-cell-defective mice lack a microenvironment supportive of o-GSC engraftment. To define the etiologies for the failure of o-GSCs to engraft in athymic mice, we demonstrate for the first time that athymic mice have impaired brain microglia/macrophage function (phagocytosis and lipopolysaccharide [LPS]-mediated cytokine production) as well as reduced expression of Ccr2 and Ccl5 relative to their wild-type counterparts. Consistent with a key role for Ccr2 and Ccl5 in gliomagenesis and optic glioma maintenance, o-GSCs do not form glioma-like lesions in either Ccr2- or Ccl5-deficient mice, and treatment with Ccl2-neutralizing antibodies attenuates Nf1 optic glioma growth in vivo. In addition, exposure of athymic mouse brain microglia to activated, but not naïve, wild-type T cells or T-cell-conditioned medium is sufficient to restore normal microglial Ccr2 and Ccl5 expression. Taken together, these observations establish an obligate role for T cells in monocyte-mediated stromal support of Nf1 LGG pathogenesis.

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Results and Discussion

**Athymic mice reveal defects in microglia/macrophages**

Because athymic mice lack Foxn1 expression, we first sought to determine whether Foxn1 loss might alter the composition of brain stromal cells previously implicated in glioma maintenance. We focused on the brain stem, as this is the second most common region for glioma formation in children with NF1 [Mahdi et al. 2017] and represents a location suitable for tumor cell engrafment studies [Chen et al. 2015]. In contrast to findings in athymic BALB/c mice [Htain et al. 1994], we observed no differences in astrocyte [GFAP+ cell] [Supplemental Fig. S1A], endothelial cell [CD34+ cell] [Supplemental Fig. S1B], oligodendrocyte precursor [Olig2+ cell] [Supplemental Fig. S1C], or myeloid cell [resident microglia and peripheral monocyte/macrophage; Iba1+ cell] [Fig. 1A] content in C57BL/6 athymic mouse brain stems relative to strain-matched wild-type counterparts.

Interestingly, athymic mice harbored an increased percentage of amoeboid-shaped Iba1+ cells [Fig. 1A], suggestive of an altered functional state. However, Foxn1 is expressed primarily in the thymus and skin [Nehls et al. 1994] and not in the mouse or human brain or MACS-isolated microglia/macrophages [Supplemental Fig. S1D–GI]. Foxn1 mutation can increase Foxp3+ regulatory T-cell content [Albuquerque et al. 2012; Oh et al. 2017], but no Foxp3+ cells were detected in either wild-type or athymic mouse brain stems [Supplemental Fig. S1H]. The absence of T cells in the naïve brain prompted a further exploration of Foxn1−/− microglia/macrophage properties.

Using Cx3cr1+GFP and Ccr2+RFP reporter mice, we and others have demonstrated that brain monocytes (Iba1+ cells) can be either resident brain microglia (Cx3cr1+CCR2+ or peripheral macrophages (Ccr2+) [Fig. 1B]. In this regard, ~95% of the brain monocytes are resident brain microglia (Cx3cr1+CCR2+), while the rest are likely peripheral macrophages (Ccr2+) [Mizutani et al. 2012].

**Figure 1.** Athymic mice reveal morphological defects in microglia. [A] Immunohistochemistry (IHC) reveals no differences in Iba1+ cell content; however, more amoeboid Iba1+ cells were found in athymic mice. [*] P < 0.0123; [ns] not significant [P = 0.05]. Bars, 40 µm. [B] Immunostaining of Cx3cr1−/− and Ccr2−/−RFP mouse brain stems reveals colocalization of Iba1 and GFP/RFP. Bars, 20 µm. [C] Reduced Nos2 (*** P < 0.0001), Tnf (*** P = 0.0051), Arg1 (** P = 0.0007), Il10 (** P = 0.0084), Tmem119 (** P = 0.0325), Cx3cr1 (** P < 0.0001), and Ccr2 expression was observed in athymic mouse brain stems. Cx3cr1−/−CCR2− brain stems were used as a negative control. [n.d.] Not detected.

**Figure 2.** Reduced microglia/macrophage gene expression in athymic mice. [A] Reduced Nos2 (*** P = 0.0138), Tnf (** P = 0.0113), Arg1 (** P = 0.0008), Il10 (** P = 0.00095), Tmem119 (** P = 0.0185), Cx3cr1 (** P = 0.0147), and Ccr2 (*** P = 0.0008) expression was detected in athymic mouse brain CD11b+ cells. [B] No difference in the percentages of CD11b+ cells in athymic versus wild-type mouse brain stems was observed. Bar, 40 µm. [C] Reduced Ifng (** P = 0.0176), Cdb80 (*** P = 0.0011), and Cdb86 (*** P = 0.0041) expression was detected in CD11b+ cells isolated from athymic mouse brains. [D] Reduced Tnfα (** P = 0.0164) and Il10 (*** P = 0.0107) was detected following LPS stimulation in athymic mouse brain microglia/macrophages. [E] Representative confocal images (left) of Iba1+ cells (green) and latex beads (blue) in mouse brain slices, with three-dimensional reconstructions (right) shown in gray and indicated by white arrows. Bars, 20 µm. [F] A lower phagocytic index was found in athymic mouse microglia/macrophages. No increase in phagocytic activity was observed in athymic mice following UDP stimulation. (*** P = 0.001, t-test comparison with their wild-type counterparts. [MRS] MRS2578, a UDP-P2yrs6 inhibitor; [ns] not significant [P ≥ 0.05].

To define the impact of Foxn1 mutation on microglia, we first examined the expression of transcripts previously implicated in microglia/macrophage function, including inflammatory mediators (Nos2, Tnf, Arg1, and Il10), chemokine receptors (Cx3cr1 and Ccr2), and genes involved in microglia/macrophage homeostasis (Cap1, Erg1, P2ry12, and Tmem119). While no differences in Cap1, Erg1, or P2ry12 expression were detected [data not shown], Nos2, Tnf, Arg1, Il10, Tmem119, Cx3cr1, and Ccr2 expression was reduced in the brain stems of athymic, relative to wild-type, mice (Fig. 1C). This reduction was also observed in acutely isolated CD11b+ cells using magnetic-activated cell sorting (MACS) [Fig. 2A], where the percentages of CD11b+ cells were similar in wild-type and athymic brain stems [Fig. 2B]. Additionally, the expression of several antigen presentation-related genes (Ifng, Cdb80, and Cdb86) was reduced in isolated athymic mouse brain microglia/macrophages relative to their wild-type counterparts [Fig. 2C].

Second, based on reduced baseline Tnf and Il10 expression in athymic mouse microglia, we next sought to determine whether Foxn1−/− microglia have impaired Tnfα and Il10 protein production following LPS stimulation. Consistent with a defect in microglia function, reduced Tnfα and Il10 levels were detected in the culture medium (CM) of athymic mouse microglia relative to their wild-type counterparts [Fig. 2D].

Third, we determined the in situ phagocytic activity of Foxn1−/− microglia. Under both basal conditions and following UDP stimulation, athymic mouse microglia...
exhibited reduced phagocytosis compared with their wild-type counterparts [Fig. 2E,F]. Whereas UDP increased phagocytosis in wild-type microglia/macrophages, which was blocked by the MRS inhibitor, the phagocytic index was lower in Foxn1−/− microglia under all conditions.

Collectively, these findings establish that Foxn1−/− brain microglia/macrophages are abnormal with respect to morphology, gene expression, and functional properties, raising the intriguing possibility that athymic mouse brain microglia are defective in their ability to provide a supportive microenvironment for o-GSC engrafment.

Reduced expression of Ccr2 in athymic mouse microglia/macrophages inhibits tumor formation

The reduction in Cx3cr1 and Ccr2 levels in athymic mouse microglia/macrophages is interesting, since Cx3CR1 and CCR2 are critical regulators of microglia/macrophage function relevant to malignant glioma growth (Feng et al. 2015; Chen et al. 2017). As such, Cx3Cr1 and Ccr2 are enriched in naïve mouse brain microglia/macrophages (Supplemental Fig. S2A), consistent with previous findings that Cx3cr1 and Ccr2 are expressed primarily by brain microglia and peripheral macrophages, respectively (Mizutani et al. 2012). Since we demonstrated previously that reduced Cx3cr1 expression delayed, but did not abrogate, murine Nf1 optic gliomagenesis (Pong et al. 2013), we specifically focused on the role of Ccr2 in o-GSC engrafment.

First, we sought to determine whether Ccr2 deficiency results in altered brain microglia morphology and gene expression. Similar to athymic mice, Ccr2−/− mice had more amoeboid Iba1+ cells [Fig. 3A], with no change in total Iba1+ cells [Supplemental Fig. S2B]. In addition, Nos2, Arg1, Il10, and Tmem119 expression was decreased in the Ccr2−/− mouse brain stems relative to their wild-type counterparts [Supplemental Fig. S2C]. Second, to assess certain whether Ccr2 is required for murine LGG formation, o-GSCs were implanted into the brain stems of wild-type and Ccr2−/− mice. While o-GSCs formed glioma-like lesions in wild-type mice 6 mo after transplantation, as evidenced by an increased percentage of Ki67+ cells at injection sites, no tumors formed in Ccr2−/− mice [Fig. 3B]. The o-GSC cells were labeled with mCherry, and mCherry+ cells were detected by immunohistochemistry (IHC) at sites where tumors formed [Supplemental Fig. S2D]. Similarly, Cx3cr1−/−/Ccr2−/− mice exhibited more amoeboid Iba1+ cells [Fig. 3C] and did not support glioma formation following o-GSC transplantation [Fig. 3D]. Third, to define the requirement for Ccr2 activation in Nf1 optic glioma maintenance, Nf1−/−/Foxn1−/−/GFAP-Cre mice [Bajenaru et al. 2003] were treated at 3 mo of age with Ccl2-neutralizing antibodies to block Ccr2 engagement. Following anti-Ccl2 treatment, the percentage of amoeboid Iba1+ cells was increased relative to the IgG-treated control group [Fig. 3E], similar to Ccr2−/− brain stems [Fig. 3A], without any changes in total Iba1+ cells [Supplemental Fig. S2E]. Importantly, mice receiving anti-Ccl2 antibody therapy had attenuated tumor proliferation [reduced BrdU+ cells] [Fig. 3F]. Taken together, these data establish that the Ccl2/Ccr2 axis is required for murine Nf1 optic gliomagenesis.

Ccl5 is necessary and sufficient for glioma formation

Tumor-associated monocytes (TAMs) can produce paracrine factors that increase glioma growth (Solga et al. 2015; Chang et al. 2016). As such, we previously used RNA sequencing of TAMs isolated from this particular murine Nf1 optic glioma model to demonstrate that microglial Ccl5 drives murine Nf1 optic glioma growth in vivo [Solga et al. 2015]. In light of the defects observed in Foxn1−/− microglia, we therefore examined Ccl5 production: At both the RNA and protein levels, Ccl5 was reduced in the brain stems of athymic mice [Fig. 4A,B]. Since Ccl5 is expressed primarily by microglia/macrophages in nonneoplastic brains [Supplemental Fig. S3A], Ccl5 expression was also reduced in Foxn1−/− MACS-isolated brain microglia [Fig. 4C].

In order to better characterize the spectrum of potential growth factors reduced in Foxn1−/− microglia, RNA sequenc- ing of the brain stems of wild-type and athymic mice was performed. Interestingly, only three growth factors (Il16, Cxcl110, and Ccl8) normally expressed in microglia/macrophages [Supplemental Fig. S3B–D] were reduced in athymic, relative to wild-type, mouse brain stems [Supplemental Fig. S3E]. These differentially expressed genes were validated by quantitative real-time PCR [qRT-PCR] using independently derived brain stem tissues and MACS-isolated microglia [Supplemental Fig. S3E]. However, no similarities in the patterns of differentially expressed genes between wild-type and athymic mouse brain stems were observed relative to those reported for microglia in the setting of inflammatory or neurodegenerative diseases [Keren-Shaul et al. 2017; Krasmann et al. 2017; data not shown].

Since Ccl5 inhibition inhibits Nf1 optic glioma growth in vivo [Solga et al. 2015], we sought to determine whether Ccl5 regulates the expression of Il16, Cxcl110, or Ccl8. Consistent with Ccl5 as a tumor microenvironment master regulator, all three genes were decreased in Ccl5−/−, relative to wild-type, mouse brain stems and MACS-isolated microglia [Supplemental Fig. S3F,G].
CD3 as a pan-T-cell marker, increased CD3+ cells were detected in murine Nf1 optic gliomas [Nf1<sup>lox/mut</sup> GFAP-Cre mice] [Fig. 5A] as well as within the glioma-like lesions following o-GSC engraftment in wild-type mice. In contrast, CD3+ cells were not found in normal brains [Fig. 5B] or o-GSC-injected sites where no tumors formed [Supplemental Fig. S4A]. Finally, consistent with reports describing increased numbers of T cells in sporadic human PAs [Yang et al. 2011; Griesinger et al. 2013], CD3+ cells were also observed in human NF1-PA (<i>n</i> = 3 tumors), with no or only rare CD3+ cells in normal brains [Fig. 5C; Supplemental Fig. S4B].

To determine whether T cells can stimulate athymic mouse brain microglia to express Ccl5, we isolated T cells from the spleens of wild-type mice and cocultured them with Foxn1<sup>−/−</sup> microglia. After 24 h, CM was collected for Ccl5 enzyme-linked immunosorbent assay [ELISA], which revealed increased Ccl5 production following exposure of athymic mouse microglia/macrophages to activated wild-type T cells relative to microglia/macrophages alone. (A) IHC reveals increased CD3+ cells in the optic nerves/chiasm of Nf1<sup>lox/mut</sup> GFAP-Cre [optic glioma], relative to Nf1<sup>lox/mut</sup>, mice [healthy optic nerves]. (**) <i>P</i> = 0.0025. Bar, 100 µm. (B) IHC reveals CD3+ cells at injected glioma-like lesion-bearing sites, but not in the contralateral sites, of wild-type mice. (n.d.) Not detectable. Bar, 20 µm. (C) IHC reveals CD3+ cells in human NF1-PA but not in the normal adjacent brain. Bar, 40 µm. (D) Wild-type T cells were cocultured with athymic mouse brain microglia/macrophages (m/m) followed by CM collection for enzyme-linked immunosorbent assay [ELISA] and CD11b+ cell capture [MACS] for qRT-PCR. (E) ELISA reveals increased Ccl5 production following exposure of athymic mouse microglia/macrophages to activated, but not naïve, wild-type T cells. (**) <i>P</i> = 0.0001. (F) Increased Ccl5 RNA expression was observed in CD11b+ microglia/macrophages exposed to activated, but not nonactivated, wild-type T cells. (**) <i>P</i> < 0.0001. (G,H) Increased Ccl5 protein [G] and RNA [H] expression in athymic mouse brain microglia/macrophages following exposure to activated wild-type T-cell CM as well as activated wild-type T cells relative to microglia/macrophages alone. (***) <i>P</i> < 0.0001; (***) <i>P</i> < 0.001. (FC) Fold change; (ns) not significant.
and microglia/macrophages were captured by MACS for Cc15 RNA quantitation using qRT-PCR [Fig. 5D]. Since productive T-cell–microglia/macrophage interactions often require T-cell activation (Chabot et al. 1999, 2002), T cells were activated with anti-CD3ε and anti-CD28 antibodies in vitro. Following activation, increased Cc15 levels were detected in the CM [Fig. 5E] as well as in MACS-isolated athymic mouse brain microglia from these cocultures [Fig. 5F]. Consistent with a model in which T-cell activation “educates” microglia/macrophages to provide a supportive microenvironment, increased Ccr2, Cx3cr1, Tmem119, Nos2, Tnf, and Arg1 expression was also found in MACS-isolated Foxn1−/−/brain microglia/macrophages following activated T-cell coculture [Supplemental Fig. S5A–F].

While T cells account for only 3%–7% of the immune cells in the naïve mouse brain [Korin et al. 2017], they could potentially interact with parenchymal cells, such as microglia. Alternatively, T-cell–microglia/macrophage interactions could take place outside of the brain. In this regard, reduced Cc15 levels were also observed in spleen monocytes isolated from athymic mice [Supplemental Fig. S5G], revealing a more pervasive defect in Foxn1−/− monocytes.

In multiple sclerosis, T-cell activation and direct T-cell–microglia/macrophage cell contact are both necessary to induce the production of soluble mediators [e.g., IFNy, IL-10, iNOS, and TNFα], which in turn alter the local microenvironment [Carson et al. 1999; Chabot et al. 1999; Dasgupta et al. 2002]. To determine whether T-cell activation is required for T cells to increase microglial Cc15 expression, athymic mouse brain microglia were grown in the presence of CM from activated or non-activated [naïve] T cells. Increased Cc15 RNA and protein expression was observed only in microglia cocultured with activated, but not nonactivated, T cells [Fig. 5E,F]. T cells can prime microglia/macrophages by direct interactions [e.g., cell membrane ligand–receptor engagement] or indirect mechanisms [e.g., secrete soluble mediators]. To ascertain whether direct cell–cell contact is required for T cells to increase microglia/macrophage Cc15 expression, Foxn1−/− brain microglia/macrophages were exposed to CM collected from activated T cells. Activated T-cell CM alone was sufficient to increase Cc15 RNA and protein expression from athymic mouse microglia/macrophages to levels similar to that observed following the addition of T cells [Fig. 5G,H]. While T-cell–microglia/macrophage interaction requires T-cell activation, but not direct cell–cell contact, future studies will be required to define the mechanisms underlying T-cell activation in these pediatric LGGs and the soluble factors responsible for mediating microglia/macrophage priming.

Taken together, we demonstrated that activated T cells secrete soluble factors that stimulate microglia to express Cc15, which provides a supportive microenvironment for o-GSC engraftment. These studies establish acquired LGG-promoting defects in Foxn1−/− microglia/macrophages that result from a failure of T-cell “education.” The inability of T cells to interact with microglia/macrophages in athymic mice may represent one barrier to successful PA patient-derived xenograft engraftment and the development of human preclinical LGG models. In addition, the instructive function of T cells in priming microglia/macrophages to create a permissive microenvironment for glioma formation and maintenance suggests additional therapeutic strategies that interrupt these important tumor ecosystem interactions.

Materials and methods

**Mice**

One-month-old male wild-type [B6-M] and athymic [Foxn1−/−; B6NU-M] C57BL/6 mice were purchased from Taconic Biosciences. Cx3cr1−/−/GFP and Ccr2−/−/RFP mice (Keiko Hirose, Washington University) were intercrossed to generate Cx3cr1−/−Ccr2−/− and Ccr2−/− mice. Cc15−/− mice were purchased from the Jackson Laboratory [005090]. Nf1−/−/GFP and Nf1−/−/RFP, GFAP-Cre mice were generated as described previously [Bajenaru et al. 2003]. All mice were maintained on a C57BL/6 background and used in accordance with an approved Animal Studies Committee protocol.

**Stem cell culture**

o-GSCs and neural stem cells were isolated and cultured as described previously [Chen et al. 2015; Pan et al. 2017] and detailed in the Supplemental Material.

**Isolation of microglia/macrophages**

Microglia/macrophages were isolated and cultured using an established protocol with modifications [Moussaoud and Draheim 2010] and as detailed in the Supplemental Material.

**T-cell culture**

Wild-type mouse spleens were homogenized in PBS containing 0.1% BSA and 0.6% Na-citrate, washed, and incubated with 120 Kunitz units of DNase I for 15 min at room temperature. Single-cell suspensions were obtained by filtering through a 30-μm strainer followed by red blood cell lysis [Erybloc, 00433357] and negative selection using the pan-T-cell isolation kit II [Miltenyi Biotec, 130-095-130]. T cells were grown in RPMI-1640 medium supplemented with 10% FBS and 1% P/S.

**In situ phagocytosis assay**

The phagocytosis assay was performed as described previously [Wendt et al. 2017] and detailed in the Supplemental Material.

**IHC**

Mice were euthanized and perfused in Ringer solution and then in 4% paraformaldehyde, and the brains and optic nerves/chiasms were dissected for IHC using primary and secondary antibodies as reported previously [Supplemental Table S1; Chen et al. 2015] and detailed in the Supplemental Material.

**Statistical analyses**

Data analyses were performed using Graphpad Prism. Unpaired two-tailed Student’s t-tests were used to determine differences between two groups. All data are presented as mean values with standard deviation. All in vitro experiments have at least three repeats with similar results. The number of biological repeats is indicated as the number of dots in the scatter plots.

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**Author contributions**

Y.P., M.X., and D.H.G. conceived the study. Y.P., M.X., R.C., Y.M., C.C., S.D., Y.-H.C., M.M., M.S., and U.K. performed the investigation. Y.P. and D.H.G. wrote the manuscript. D.H.G. supervised the study.
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