CCR2 inhibition reduces tumor myeloid cells and unmasks a checkpoint inhibitor effect to slow progression of resistant murine gliomas


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Immunotherapy directed at the PD-L1/PD-1 axis has produced treatment advances in various human cancers. Unfortunately, progress has not extended to glioblastoma (GBM), with phase III clinical trials assessing anti-PD-1 monotherapy failing to show efficacy in newly diagnosed and recurrent tumors. Myeloid-derived suppressor cells (MDSCs), a subset of immunosuppressive myeloid derived cells, are known to infiltrate the tumor microenvironment of GBM. Growing evidence suggests the CCL2–CCR2 axis is important for this process. This study evaluated the combination of PD-1 blockade and CCR2 inhibition in anti-PD-1-resistant gliomas. CCR2 deficiency unmasked an anti-PD-1 survival benefit in KR158 glioma-bearing mice. CD11b+Ly6C+PD-L1+ MDSCs within established gliomas decreased with a concomitant increase in overall CCR2+ cells and MDSCs within bone marrow of CCR2-deficient mice. The CCR2 antagonist CCX872 increased median survival as a monotherapy in KR158 glioma-bearing animals and further increased median and overall survival when combined with anti-PD-1. Additionally, combination of CCX872 and anti-PD-1 prolonged median survival time in 005 GSC GBM-bearing mice. In both models, CCX872 decreased tumor associated MDSCs and increased these cells within the bone marrow. Examination of tumor-infiltrating lymphocytes revealed an elevated population, increased IFNγ expression, indicating enhanced cytolytic activity, as well as decreased expression of exhaustion markers in CD4+ and CD8+ T cells following combination treatment. These data establish that combining CCR2 and PD-1 blockade extends survival in clinically relevant murine glioma models and provides the basis on which to advance this combinatorial treatment toward early-phase human trials.

Significance

Standard of care for glioblastoma (GBM) results in median survival of <15 mo with 5-y survival <10%, highlighting the need for novel treatments. Immune checkpoint blockade has been successful in various cancer subtypes. However, success has not extended to GBM, largely due to the characteristically immune-suppressive glioma microenvironment. Here, we report targeting of myeloid derived suppressor cells (MDSCs), via inhibition of CCR2, unmasks efficacy of checkpoint blockade in GBM. Using checkpoint inhibitor-resistant murine glioma models, we show combination treatment reduces MDSCs and increases functional T cells within the tumors, and significantly extends overall survival. Our results support targeting of CCR2-expressing myeloid cells to enhance immunotherapies for GBM, and warrant translation of this approach to early human clinical trials.


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against either programmed death-1 or its ligand, i.e., PD-1/PD-L1. Tumor cells exploit the PD-1/PD-L1 axis as a means to evade immune surveillance by expressing PD-L1. The receptor for PD-L1, PD-1, is expressed by T cells and, when stimulated, acts to inhibit T cell activation/effector function. The use of checkpoint inhibitors in certain cancers such as melanoma (17, 18), lung (19), renal cell carcinoma (20), colon (21), and hepatocellular carcinoma (22, 23) has proven effective. Although intratumoral expression of PD-L1 in GBM has been observed, correlations with patient outcome have been inconclusive or inconsistent (24–26). Despite discrepancy in the prognostic ability of PD-L1 expression, these studies posit that the use of immune-checkpoint inhibitor-based approaches is warranted, although this line of therapy has failed as a single treatment in recurrent and newly diagnosed GBM. While the reasons for these incomplete response rates are poorly understood, it has been shown that a small subset of GBM patients presenting with higher than typical mutational load respond well to anti-PD-1 therapy (27). The success of PD-1 blockade in these patients suggests checkpoint blockade may be a viable therapy for GBM. Moreover, the recent demonstration that neoadjuvant PD-1 blockade provided a benefit for GBM patients over adjuvant therapy has renewed interest in immune-checkpoint inhibitor therapy in GBM (13, 14). Preclinical studies aimed at augmenting the efficacy of checkpoint blockade via combinatorial approaches targeting the immune suppressive microenvironment are ongoing (28–32).

A subset of myeloid-derived cells with immunosuppressive properties, termed myeloid-derived suppressor cells (MDSCs), are known to be elevated in the peripheral blood of GBM patients (33). MDSCs infiltrate the glioma microenvironment, and act as drivers of the immune-suppressive phenotype typical of these tumors (33–42). The mechanism by which they traffic to the tumor site is not entirely clear, although growing data suggest chemokine receptors are important for this process, namely chemokine receptor 2 (CCR2) (40, 43–48). Indeed, previous reports indicate Ly6C<sup>+</sup> inflammatory monocytes express CCR2 (43). Additionally, known ligands of this receptor (CCL2 and CCL7) are expressed by gliomas and are thought to mediate tumor recruitment of CCR2<sup>+</sup> cells (40, 44, 47, 49, 50). Approaches that specifically target the MDSC population have been employed in various tumor types where they have been shown to enhance immune response. In some cases, even reverse resistance to checkpoint blockade (51–55).

In this study, we sought to more clearly define the role of CCR2 in immune-checkpoint inhibitor-resistant high-grade gliomas. Using CCR2-deficient mice, we report loss of CCR2 expression prevented egress of MDSCs from the bone marrow, reduced glioma infiltration of these cells, and unmasked an anti-tumor effect of PD-1 blockade. Further, use of a CCR2 antagonist, CCX872, recapitulated the findings of genetic ablation, demonstrating reductions in MDSC glioma infiltration and imparting an enhanced anti-PD-1 survival effect. Taken together, we suggest that disruption of the CCL2/CCR2 axis promotes sequestration of CCR2<sup>+</sup> cells within bone marrow, limiting distribution of these immune-suppressive cells to the tumor, and allowing for attenuation of the immunosuppressive tumor microenvironment. As such, the amelioration of immune suppression via CCR2 inhibition may contribute to enhanced anti-PD-1 efficacy in gliomas.

### Results

**Distinct CCR2- and CX3CR1-Expressing Myeloid Cell Populations in Glioma-Bearing Mice.** CCR2<sup>+</sup> cells do not represent the sole myeloid cell type present in gliomas, as CX3CR1<sup>+</sup> central nervous system (CNS)-resident microglia are known to infiltrate as well. As a means to investigate the glioma presence of these chemokine receptor-expressing myeloid cell populations, we employed double transgenic mice that carry RFP in place of the CCR2 gene (CCR2<sup>RFP/WT</sup>) and GFP in place of CX3CR1 (CX3CR1<sup>GFP/WT</sup>) as knock-in alleles, enabling direct surveillance of CCR2<sup>+</sup> and CX3CR1<sup>+</sup> cells. Two therapy-resistant murine glioma models were employed, including the high-grade glioma KR158 model and the recently reported GBM stem-like cell 005 GSC model (56–58). These models exhibit common histological characteristics of glioma, including areas of increased cell density, necrotic regions within the tumor, invasion of normal brain parenchyma, and pseudopalisading necrosis (SI Appendix, Fig. S1 A–C), consistent with previously published results (56).

Fluorescent imaging confirmed the presence of both CCR2<sup>+</sup> and CX3CR1<sup>+</sup> cells within KR158 tumors (Fig. 1A). Flow-cytometric analysis identified tumor-associated CCR2<sup>+</sup> and CX3CR1<sup>+</sup> cells in both glioma models. However, the presence of both populations was significantly higher in KR158 tumors (CCR2<sup>+</sup>, P = 0.048; CX3CR1<sup>+</sup>, P = 0.012) (Fig. 1B). Analysis of the bone marrow revealed a significant increase in CCR2<sup>+</sup> cells upon either KR158 (P = 0.032) or 005 GSC (P = 0.001) tumor implantation, with no change in this cell population as a result of phosphate-buffered saline (PBS) injection (Fig. 1C). The GFP<sup>+</sup>/RFP<sup>+</sup> cell population (CCR2<sup>+</sup>/CX3CR1<sup>+</sup>) was unchanged in the bone marrow of the tumor-bearing animals.

We next sought to characterize the myeloid marker phenotypes of the CCR2<sup>+</sup> and CX3CR1<sup>+</sup> populations in the tumor microenvironment. In order to investigate these populations, tumor infiltrates from glioma-bearing CCR2<sup>RFP/WT</sup>/CX3CR1<sup>GFP/WT</sup> mice were subjected to flow-cytometric analysis of CD45, CD11b, Ly6C, and Ly6G. Two distinct CD45<sup>+</sup> populations were identified, designated CD45<sup>high</sup> and CD45<sup>low</sup> (Fig. 1D). Analysis of these populations revealed CD45<sup>low</sup> events (Fig. 1D, Upper) represent a cell population that is primarily CX3CR1<sup>+</sup>, likely representing microglia. CD45<sup>high</sup> (Fig. 1D, Middle) events represent a more heterogeneous cell population consisting of CCR2<sup>+</sup>, CX3CR1<sup>+</sup>, and CCR2<sup>+</sup>/CX3CR1<sup>+</sup> cells. Murine monocytes MDSCs are typically classified as CD11b<sup>+</sup>/Ly6C<sup>+</sup>/Ly6G<sup>+</sup>. To examine the heterogeneous CD45<sup>+</sup> population, we scrutinized CCR2<sup>+</sup> and CX3CR1<sup>+</sup> populations by expression of CD11b/Ly6C/Ly6G. Flow-cytometric analysis of Ly6C/Ly6G noted 3 distinct Ly6C populations: negative, intermediate, and high (Fig. 1E). Ly6G expression was minimal in the tumors. Ly6C<sup>+</sup> events (Fig. 1, E, Upper) represented a cell population that is primarily CCR2<sup>+</sup>/CX3CR1<sup>+</sup>, while Ly6C<sup>–</sup>/Ly6G<sup>+</sup> (Fig. 1, E, Middle) events consist of CCR2<sup>+</sup>/CX3CR1<sup>+</sup>, and CCR2<sup>+</sup>/CX3CR1<sup>+</sup> cells. Ly6C<sup>+</sup>/Ly6G<sup>+</sup> events were determined to be CCR2<sup>+</sup>/CX3CR1<sup>+</sup> double-positive (SI Appendix, Fig. S2B). Similar analysis within bone marrow isolates revealed 4 distinct populations: negative, Ly6C<sup>+</sup>/Ly6G<sup>+</sup>/Ly6C<sup>+</sup>/Ly6G<sup>+</sup> (SI Appendix, Fig. S2C). Ly6C<sup>+</sup>/Ly6G<sup>+</sup> events were primarily CCR2<sup>+</sup>/CX3CR1<sup>+</sup>, while Ly6C<sup>+</sup>/Ly6G<sup>+</sup> (SICCR2<sup>+</sup>/CX3CR1<sup>+</sup> double-positive (SI Appendix, Fig. S3 A–D). Taken together, invading myeloid cells expressing the 2 chemokine receptors within the tumor microenvironment are predominantly CCR2<sup>+</sup> or CCR2<sup>+</sup>/CX3CR1<sup>+</sup> double-positive, while resident myeloid-like cells are predominantly CX3CR1<sup>+</sup>.

**CCR2 Deficiency Unmasks an Anti-PD-1 Effect in Immune-Checkpoint Inhibitor-Resistant Glioma.** To establish a role of CCR2 in glioma and the potential impact of disrupting this receptor on the efficacy of immune-checkpoint inhibitors, we evaluated the effect of anti-PD-1 monotherapy in CCR2-deficient and −/− mice. In KR158 tumor-bearing (n = 8 to 10/group) CCR2<sup>RFP/WT</sup> or CCR2<sup>RFP/WT</sup> mice, we dosed with anti-PD-1 starting at day 7, as
Flow-cytometric analysis of tumor isolates from Ccr2RFP/WT;Cx3cr1GFP/WT mice. The left graphs represent Ly6C+ tumor-bearing CCR2-deficient mice (reported as pixel density vs. area of the cross-section) in non-tumors from CCR2-deficient mice. Fluorescence imaging of these samples (Fig. 2) shows CX3CR1+ events represent a cell population that is primarily CCR2−/CX3CR1+ subpopulations: negative (bottom), intermediate (middle), and high (top). Blue arrows denote subpopulations plotted by expression of CCR2 and CX3CR1.

Quantification shows increase in CCR2 single-positive cells in KR158 (P = 0.012) and 005 GSC (P = 0.001) tumor-bearing animals. (D) Flow-cytometric analysis of tumor isolates from Ccr2RFP/WT;Cx3cr1GFP/WT normal (N) and tumor (T) tissue. Red fluorescence denotes CCR2+ cells, while green fluorescence denotes CX3CR1+ cells. (Image magnification: 20×.) (B) Flow-cytometric analysis of tumor isolates from KR158 (n = 4) (Left) and 005 GSC (n = 3) (Right) tumor-bearing Ccr2RFP/WT;Cx3cr1GFP/WT mice. Higher CCR2 single-positive (P = 0.048) and CX3CR1 single-positive (P = 0.012) cell populations in KR158 vs. 005 GSC glioma models are noted. (C) Flow-cytometric analysis of bone marrow cell populations in CCR2/RFP vs. CX3CR1/GFP in naive (n = 3) (Upper Left), mock PBS-injected (n = 6) (Upper Right), 005 GSC (n = 3) (Lower Left), and KR158 (n = 6) (Lower Right) tumor-bearing Ccr2RFP/WT;Cx3cr1GFP/WT animals. Quantification shows increase in CCR2 single-positive cells in KR158 (P = 0.032) and 005 GSC (P = 0.001) tumor-bearing animals. (D) Flow-cytometric analysis of tumor isolates from Ccr2RFP/WT;Cx3cr1GFP/WT mice. The left graphs represent forward scatter (FSC) vs. CD45 plots demonstrating 3 CD45 populations: negative (left), low (middle), and high (right). Blue arrows denote subpopulations plotted by expression of CCR2 and CX3CR1. CD45+ events (Upper) represent a primarily CX3CR1+ cell population, while CD45+ events represent a heterogeneous cell population consisting of CCR2−, CX3CR1+, and CCR2+/CX3CR1− cells. (E) Flow-cytometric analysis of tumor isolates from Ccr2RFP/WT;Cx3cr1GFP/WT mice. The left graphs represent Ly6C+ vs. Ly6C− events and demonstrate 3 Ly6C populations: negative (bottom), intermediate (middle), and high (top). Blue arrows denote subpopulations plotted by expression of CCR2 and CX3CR1. Ly6C+ events represent a cell population that is primarily CCR2+/CX3CR1−, while Ly6C− events represent a heterogeneous cell population consisting of CCR2−, CX3CR1+, and CCR2+/CX3CR1− cells. Representative plots are shown throughout. *P < 0.05; **P < 0.01.

Fig. 1. Distinct cell populations of CCR2- and CX3CR1-expressing myeloid cells in glioma-bearing mice. (A) Fluorescent images showing representative example of section of KR158 tumor-bearing Ccr2RFP/WT;Cx3cr1GFP/WT normal (N) and tumor (T) tissue. Red fluorescence denotes CCR2+ cells, while green fluorescence denotes CX3CR1+ cells. (Image magnification: 20×.) (B) Flow-cytometric analysis of tumor isolates from KR158 (n = 4) (Left) and 005 GSC (n = 3) (Right) tumor-bearing Ccr2RFP/WT;Cx3cr1GFP/WT mice. Higher CCR2 single-positive (P = 0.048) and CX3CR1 single-positive (P = 0.012) cell populations in KR158 vs. 005 GSC glioma models are noted. (C) Flow-cytometric analysis of bone marrow cell populations in CCR2/RFP vs. CX3CR1/GFP in naive (n = 3) (Upper Left), mock PBS-injected (n = 6) (Upper Right), 005 GSC (n = 3) (Lower Left), and KR158 (n = 6) (Lower Right) tumor-bearing Ccr2RFP/WT;Cx3cr1GFP/WT animals. Quantification shows increase in CCR2 single-positive cells in KR158 (P = 0.032) and 005 GSC (P = 0.001) tumor-bearing animals. (D) Flow-cytometric analysis of tumor isolates from Ccr2RFP/WT;Cx3cr1GFP/WT normal (N) and tumor (T) tissue. Red fluorescence denotes CCR2+ cells, while green fluorescence denotes CX3CR1+ cells. (Image magnification: 20×.) (E) Flow-cytometric analysis of tumor isolates from Ccr2RFP/WT;Cx3cr1GFP/WT mice. The left graphs represent forward scatter (FSC) vs. CD45 plots demonstrating 3 CD45 populations: negative (left), low (middle), and high (right). Blue arrows denote subpopulations plotted by expression of CCR2 and CX3CR1. Ly6C+ events represent a cell population that is primarily CCR2+/CX3CR1−, while Ly6C− events represent a heterogeneous cell population consisting of CCR2−, CX3CR1+, and CCR2+/CX3CR1− cells. Representative plots are shown throughout. *P < 0.05; **P < 0.01.

CCKR2 Deficiency Has Reciprocal Effects on Presence of MDSCs in Tumor and Bone Marrow. Imaging analysis of CCR2 promoter-driven RFP staining for the myeloid marker CD11b confirmed the presence of CCKR2+ myeloid derived cells within KR158 gliomas (Fig. 2B). The presence of these cells was reduced in KR158 tumors from CCKR2-deficient mice. Fluorescence imaging of bone marrow revealed significantly elevated CCKR2/RFP signal (reported as pixel density vs. area of the cross-section) in non-tumor-bearing CCKR2-deficient mice (P = 0.029) as compared to CCKR2-sufficient animals. Further elevation was observed in both CCKR2+/F+F/PWT (P = 0.011) and CCKR2+/F+F/PFP (P = 0.036) following KR158 tumor implantation (Fig. 2C).

Flow-cytometric analysis of the tumor-associated RFP+ cell population revealed a statistically significant decrease (P = 0.047) of this population, while similar analysis of bone marrow showed a significant increase (P = 0.024) (Fig. 3A) in CCKR2-deficient tumor-bearing mice. Not all CCKR2+ cells were found to be Ly6C+. In order to more accurately examine the effect of CCKR2 deficiency on the immune-suppressive cell population of these mice, flow-cytometric analysis of immune cells isolated from tumors and bone marrow of CCKR2+/F+F/PWT and CCKR2+/F+F/PFP mice was performed. Analysis revealed a statistically significant reduction (P = 0.039) of MDSCs (CD45−/CD11b+/Ly6C+) within KR158 tumors with a concomitant increase (P = 0.020) in bone marrow (Fig. 3B). Additionally, investigation of this population in the periphery was performed, and a significant reduction (P = 0.048) in the MDSC population present within spleens of tumor-bearing animals was evident (SI Appendix, Fig. S4A). The proportion of RFP+ cells that are also Ly6C+ within the bone marrow is unchanged by CCKR2 deficiency (Fig. 3C). However, when this proportion was determined in tumors, a marked reduction (P = 0.007) of this population was noted with CCKR2 deficiency.

It has been reported that MDSCs residing within the tumor microenvironment prevent the entry of CD8+ T cells into the
tumor (59). Despite a noted reduction in MDSCs within tumors, an increase in CD4+ T cells (P = 0.031) was observed, while the population of CD8+ T cells remained unaltered by CCR2 knockout (SI Appendix, Fig. S5B). A significant increase (P = 0.003) of the ratio of CD8+ T cells/MDSCs was evident within tumors derived from CCR2-deficient mice (SI Appendix, Fig. S5C).

CCR2 Antagonist CCX872 Enhances an Anti-PD-1 Effect to Improve Survival. Given the enhanced anti-PD-1 effect in CCR2-deficient KR158 tumor-bearing mice, we hypothesized that pharmacological antagonism of CCR2 would augment the efficacy of immune-checkpoint blockade. To test this hypothesis, we evaluated the effect of an orally active, high-affinity CCR2 antagonist, CCX872, on glioma-bearing mice. Due to the enhanced CCR2/RFP signal in bone marrow of naive mice (P = 0.036), which was further enhanced in tumor-bearing CCR2RFP/RFP animals (P = 0.036), we assessed the relationship between tumor-infiltrating CD8+ T cells and CCR2 expression (Fig. 4A). CD8+ T cells/MDSCs was evident within tumors derived from CCR2-deficient mice (SI Appendix, Fig. S5C).

CX3CR1+/Ly6C+ cells within the tumors, and a concomitant increase (P = 0.028) of this population in the bone marrow was seen (Fig. 5B).

We next investigated the effect of CCX872 treatment on the 3 CCR2- and CX3CR1-expressing subpopulations. KR158 or 005 GSC bearing CCR2RFP/WT,CX3CR1GFP/WT mice were treated with either vehicle or CCX872. Immune cell populations were subsequently isolated and subjected to flow-cytometric analysis of CCR2/RFP and CX3CR1/GFP expression, as well as for CD45, CD11b, Ly6C, and Ly6G. Analysis of KR158 tumors revealed a significant decrease (P = 0.003) in RFP+, i.e., CCR2+/CX3CR1− cells with CCX872 treatment. Similarly, CCR2+/CX3CR1+ reported a decrease (P = 0.032) with CCX872 treatment (Fig. 5C, Upper). Consistent with previous results, CCX872 treatment reduced (P = 0.004) CD45b+CD11b+Ly6C+ cells within KR158 tumors (Fig. 5C, Lower). Parallel analysis was performed in 005 GSC glioma-bearing animals. A significant reduction of CCR2 single-positive (P = 0.003), CX3CR1+ (P = 0.003), as well as CCR2/CX3CR1 double-positive (P = 0.042) events (Fig. 5D, Upper) were observed in tumors from CCX872-treated mice. Analysis of CD45b+CD11b+Ly6C+ cells within 005 GSC tumors also showed a reduction (P = 0.020) in Ly6C+ cells with CCX872 treatment (Fig. 5D, Lower).

CX3CR1+/Ly6C+ cells were infected with virus encoding a lentiviral shRNA against CX3CR1 (LV-shCX3CR1). The lentiviral particles were administered via intratumoral injection 1 week before CCX872 treatment. The combination of CCX872 and LV-shCX3CR1 produced a significant increase (P = 0.042) in CD8+ T cells/MDSCs, and a concomitant decrease (P = 0.004) in CD45b+CD11b+Ly6C+ cells within KR158 tumors (Fig. 5D, Lower).

Discussion

Since the inclusion of temozolomide into the standard-of-care regimen for GBM, little progress has been made in the development of effective treatments for this disease. Stagnating survival rates underscore the need for next generation approaches for the treatment of GBM. While immunotherapy-based approaches have been attempted, most clinical trials involving these modalities have failed to report significant outcomes. Elevated levels of MDSCs, both in the peripheral blood and tumor microenvironment of GBM patients, have been described (34, 39). MDSCs are known to potentiate immune suppression in GBM and may contribute to the failure of immune therapies for gliomas (34–40, 42). The exact mechanism by which MDSCs traffic to the tumor is not wholly defined, although evidence supports a role of CCR2 in this process (40). As such, we set out to determine if mice bearing immune-checkpoint inhibitor-resistant gliomas would gain a...
increased microglial infiltration in gliomas has been associated with therapeutic strategies that aim to mitigate their protumorigenic effects in glioma progression will be critical in the development of novel therapeutics. Understanding specific roles of the various myeloid cell subsets will aid in the selection of appropriate targets for intervention.

Increased CCR2 expression and marrow-derived infiltrating monocytes potentially exerting tumor growth-promoting activities have been observed. The expression of CCR2 has been shown to be integral in the migration of CCR2-expressing leukocytes, including microglia and macrophages. The receptor is known to be involved in the recruitment of leukocytes from the bone marrow to the tumor microenvironment, where they can exert pro-inflammatory effects.

Selection of KR158 and 005 GSC glioma models allowed for the evaluation of our proposed treatment paradigm in immune-competent mice that recapitulate many of the features of human GBM. Our data suggest that the enhanced survival is a consequence of reduced MDSCs within the glioma microenvironment. A concomitant increase of this cell population within bone marrow, and an increase in functional tumor-infiltrating lymphocytes, further support this conclusion.

Disruption of CCR2 not only leads to reduced MDSCs within tumors but also an associated accumulation of these cells in the bone marrow. A role for CCR2 in mobilization of leukocytes from the bone marrow has been reported (43). In this study, we identified a CX3CR1+Ly6CChigh that is present within the glioma that may impact response to treatment, as cells with similar phenotype have been linked to treatment resistance in response to anti-VEGFR2 inhibitors (65).

Previous work using the CCR2RFP/WT;CX3CR1GFP/WT double-transgenic reporter mouse demonstrated the presence of 3 CCR2- and/or CX3CR1-expressing cell populations in GL261 tumors (63), and our findings extend this observation to more clinically relevant murine models of therapy resistant gliomas. Tumor-associated myeloid (TAM) cell infiltration is an established characteristic of GBM and has a known influence on disease progression. While both resident microglia and bone marrow-derived infiltrating monocytes potentially exerting tumor growth-promoting activities have been associated with poorer outcomes in patients. Modulation of the chemokine receptor characteristically expressed by microglia, CX3CR1, by our laboratory and others reported modest reductions in survival time with CX3CR1 deficiency in murine models of glioma (43, 45). An indirect effect of CX3CR1 deficiency on the presence of MDSC-like monocytes within CNS tumors and an associated increase of IL1β and the CCR2 ligand, CCL2, has been reported (43). In this study, we identified a CX3CR1+Ly6CChigh that is present within the glioma that may impact response to treatment, as cells with similar phenotype have been linked to treatment resistance in response to anti-VEGFR2 inhibitors (65). Furthermore, Butowski et al. (66) investigated the efficacy of using a colony-stimulating factor 1 receptor (CSF1R) inhibitor as a means to modulate microglia within the GBM tumor microenvironment but found no clinical benefit. This approach of broadly targeting CSF1R-expressing myeloid populations, which include microglia, macrophages, mast cells, and osteoclasts, may limit overall efficacy within the CNS. Additionally, impacting brain-derived microglia and/or myeloid cells after they gain entry into the tumor requires drug penetration of the blood–brain barrier. The approach reported herein may hold value for more specific intervention by antagonizing only the CCR2-expressing subpopulation of myeloid cells. Furthermore, retention of MDSCs in the bone marrow as a consequence of CCR2 disruption does not require drug penetration of the blood–brain barrier.

Disruption of CCR2 not only leads to reduced MDSCs within tumors but also an associated accumulation of these cells in the bone marrow. A role for CCR2 in mobilization of leukocytes from the bone marrow has been reported previously (48, 67–69), and the mechanism likely involves interactions with another chemokine receptor, CXCR4 (70). The egress of CCR2+ cells from the bone marrow and influx into the tumors may be mediated by any known ligand for CCR2. In addition to CCL2, MCP-3 (CCL7) has been shown to be integral in migration of CCR2+ monocytes out of the bone marrow (48). Expression of CCL2 has been

**Fig. 3.** Impact of Ccr2 deficiency on peripheral and tumor MDSC populations. (A) Flow-cytometric analysis of RFP+ events in Ccr2RFP/WT (n = 6) vs. Ccr2RFP/RFP (n = 6) mice. Population of RFP+ cells within the tumor microenvironment (Upper) is reduced (P = 0.047) but increased (P = 0.024) in bone marrow (Lower) of Ccr2-deficient animals. (B) Flow-cytometric analysis of CD45+CD11b+Ly6C+ events in Ccr2RFP/WT (n = 5) vs. Ccr2RFP/RFP (n = 5) mice. Population of CD45+/CD11b+/Ly6Chigh cells within the tumor microenvironment (Upper) was reduced (P = 0.039) but increased (P = 0.020) in bone marrow (Lower) of Ccr2-deficient animals. (C) Quantification of percentage of RFP+ cells that are CD45+, CD45+/CD11b+, and CD45+/CD11b+/Ly6C+ within bone marrow (Upper) and tumor (Lower) in Ccr2RFP/WT (n = 5) vs. Ccr2RFP/RFP (n = 5) mice. Ratios remain unchanged in bone marrow but show a significant reduction (P = 0.007) of CD45+/CD11b+/Ly6Chigh cells in tumors of Ccr2RFP/RFP vs. Ccr2RFP/WT mice. Representative plots are shown throughout. *P < 0.05; **P < 0.01. FSC, forward scatter.
shown to correlate with survival time in human GBM patients, with patients who exhibit low CCL2 expression surviving longer than individuals expressing high levels of this chemokine (40). This trend was recapitulated in GL261 glioma-bearing mice (47, 63). Considering the heterogeneity in CCL2 expression observed in human patients, the variation in myeloid cell infiltration and survival between KR158 and 005 GSC CCR2 antagonist-treated gliomas noted in our study may reflect differences in CCR2 ligand expression between the 2 models. Nonetheless, given the redundancy in ligands for CCR2, approaches targeting either CCL2 or CCL7 individually may not be fruitful (71), warranting redundancy in ligands for CCR2, approaches targeting either CCL2 or CCL7 individually may not be fruitful (71), warranting redundancy in ligands for CCR2. To summarize, our data show that CCR2 deficiency augments anti-PD-1 treatment and unmask survival advantage in glioma-bearing mice. These results are recapitulated with CCR2 antagonist in mice bearing either KR158 or 005 GSC murine glioma models, supporting the hypothesis that CCR2 antagonist, when delivered in conjunction with anti-PD-1, may be a viable approach for the treatment of human gliomas. The use of anti-PD-1–resistant syngeneic murine models enhances the translational value of this study as compared to others that have relied on immune-deficient mice or anti-PD-1–responsive glioma models. Furthermore, the use of CCX872 (currently in Phase Ib clinical trials for the treatment of pancreatic cancer [ClinicalTrials.gov Identifier NCT02345408]), in combination with a clinically available treatment (PD-1 blockade) would allow for expedited translation of these preclinical results into early-phase human clinical trials.

Materials and Methods
Cell Culture. KR158 glioma cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine

Fig. 4. Effect of combinatorial CCX872/anti-PD-1 treatment on survival of KR158 and 005 GSC glioma-bearing mice. (A) Schematic representation of CCX872 and anti-PD-1 treatment schedules. Survival analysis of KR158 (n = 8 to 10) (B) and 005 GSC (n = 8 to 10) (C) tumor-bearing WT mice treated with CCX872 and anti-PD-1. In KR158 glioma-bearing mice, CCX872 increased median survival (P < 0.002, 32 vs. 50 d). Combinatorial treatment increased durable survival (P = 0.001); 005 GSC-bearing animals had an increase in median survival (P = 0.005, 30 vs. 49 d) with combinatorial treatment. Triangles mark anti-PD-1 administration. The bracket indicates CCX872 administration. *P < 0.05; **P < 0.01.
Fig. 5. Impact of combinatorial CCX872/anti-PD-1 treatment on peripheral and tumor myeloid cell populations. (A) Flow-cytometric analysis of Ly6C* vs. Ly6G* events in KR158 tumor isolates (Upper) and bone marrow cell populations (Lower) from vehicle-treated (n = 6) and CCX872-treated (n = 6) animals. Drug treatment resulted in a reduction (P = 0.038) of Ly6C* events within tumor and an increase (P = 0.028) in bone marrow. (B) Flow-cytometric analysis of Ly6C* vs. Ly6G* events in 005 GSC tumor isolates (Upper) and bone marrow cell populations (Lower) from vehicle-treated (n = 6) and CCX872-treated (n = 5) animals. Drug treatment resulted in a reduction (P = 0.015) in Ly6C* events within tumors and an increase (P = 0.028) in bone marrow. (C) Flow-cytometric analysis of tumor isolates from KR158 tumor-bearing Ccr2RFP/WT/Cx3cr1GFP/WT mice depicting CCR2* vs. CX3CR1* (Upper) and Ly6C* vs. Ly6G* events (Lower) from vehicle-treated (n = 7) and CCX872-treated (n = 7) animals. Drug treatment resulted in a significant reduction of CCR2* (P = 0.024) and CX3CR1* (P = 0.032) events. The lower graphs report a reduction (P = 0.004) in Ly6C* events within tumors. (D) Flow-cytometric analysis of tumor isolates from 005 GSC tumor-bearing Ccr2RFP/WT/Cx3cr1GFP/WT mice depicting CCR2* vs. CX3CR1* (Upper) and Ly6C* vs. Ly6G* events (Lower) from vehicle-treated (n = 6) and CCX872-treated (n = 6) animals. Drug treatment resulted in a reduction of CCR2* (P = 0.003), CX3CR1* (P = 0.003), and CCR2*/CX3CR1* (P = 0.0419) events. The lower graphs report a reduction (P = 0.020) in Ly6C* events within tumors. Representative plots are shown throughout. *P < 0.05, **P < 0.01.
Fig. 6. Impact of combinatorial CCX872/anti-PD-1 treatment on CD4+ and CD8+ T cells. (A) Flow-cytometric analysis of CD45+/CD3+/CD4+ and CD8+ events within tumor extracts from vehicle/IgG-treated (n = 7), CCX872/IgG-treated (n = 4), vehicle/anti-PD-1–treated (n = 6), or CCX872/anti-PD-1–treated (n = 4) 005 GSC glioma-bearing mice. The population of CD45+/CD3+/CD4+ cells (upper square) was significantly increased (P = 0.044) with combination CCX872/anti-PD-1 treatment as compared to vehicle/IgG, while the CD45+/CD3+/CD8+ population (lower square) trended toward increase (P = 0.056) between the same groups. (B) Flow-cytometric analysis of side scatter (SSC) vs. CD45+/CD3+/IFNα+ events (denoted by square) within tumor extracts from vehicle/IgG-treated (n = 7), CCX872/IgG-treated (n = 4), vehicle/anti-PD-1–treated (n = 5), or CCX872/anti-PD-1–treated (n = 6) 005 GSC glioma-bearing mice. The population of CD45+/CD3+/IFNα+ cells was significantly increased (P = 0.008) with combination CCX872/anti-PD-1 treatment as compared to vehicle/IgG. (C and D) Flow-cytometric analysis of CD45+/CD3+/PD-1+ and Tim3+ with CD8+ (D) events within tumor extracts from vehicle/IgG-treated (n = 7), CCX872/IgG-treated (n = 4), vehicle/anti-PD-1–treated (n = 6), or CCX872/anti-PD-1–treated (n = 4) 005 GSC glioma-bearing mice. The population of CD45+/CD3+/PD-1+/Tim3+/CD4+ cells (circled population) was significantly decreased (P = 0.029) with combination CCX872/anti-PD-1 treatment as compared to vehicle/IgG. The population of CD45+/CD3+/PD-1+/Tim3+/CD8+ cells (circled population) also decreased (P = 0.011) between the same groups. Representative plots are shown throughout. *P < 0.05, **P < 0.01.

Constant agitation. Following fixation, femurs were decalcified using 14% ethylenediaminetetraacetic acid (EDTA) and 9% ammonium hydroxide (wt/vol; pH 7.1) decalcifying solution at 4 °C for 3 d with constant agitation, changing solution every 24 h. Bones were then washed in PBS for 2 h and then soaked in 30% sucrose at 4 °C overnight with constant agitation. Bones were then embedded in optimal cutting temperature medium, sectioned, and cut on a cryotome using 10-μm sections. Sections were then washed 3 times with PBS and collected in 1.5-mL microcentrifuge tubes. Tumors were then excised from brains and minced using a razor blade. Tissue was suspended in 4 °C Accumax dissociation solution (Innovative Cell Technologies) and incubated at 37 °C for 5 min, followed by 5 min of agitation at room temperature. Cells were then washed through a 70-μm strainer, centrifuged (4 °C, 380 × g, 5 min), and resuspended in 4 mL of 70% Percoll (70% Percoll and 1% PBS in RPMI-1640 cell medium). This cell suspension was then gently layered beneath a 37% Percoll layer (4 mL, 37% Percoll and 1% PBS in RPMI-1640 cell medium) using an 18-gauge needle and centrifuged (30 min, room temperature, 500 × g), and the interface was removed and placed into a 1.5-mL microcentrifuge tube. All cells were then washed with ice-cold PBS, counted by trypan blue exclusion, and aliquoted to 1 × 10^6 cells/100 μL and blocked using 0.5%–0.5% mouse C16D62 (101320; Biolegend) for 30 min at 4 °C. Subsequently, cells were stained for markers of interest (see SI Appendix, Table S1 for antibodies used) for 30 min at 4 °C. Cells were then washed twice in ice-cold PBS and either fixed in 4% PFA for 30 min and resuspended in FACs buffer or left unfixed if isolated from reporter mice. Intracellular staining of FoxP3 was carried out according to manufacturer instructions using the eBioscience F01012103/Transcription Factor Staining Buffer Set (no. 00-5523-00; Invi-trogen). Stained samples were analyzed using single-color compensation on either a BD LSR Fortessa flow cytometer (BD Biosciences) or a Sony SP6800 spectral analyzer and quantified using FCS Express software (De Novo Software).

Statistical Analysis. Student t test was performed in SigmaPlot (SmyGo) as indicated in the results. P values were calculated using Student t test with 2-tailed distribution. Survival data were subjected to log-rank test using GraphPad Prism 5 software (GraphPad Software). To determine statistically significant differences between groups, a P value of <0.05 was considered significant and is indicated by symbols depicted in the figures and figure legends.

Data Availability. All data are included in the main text and SI Appendix.
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