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THE CCL2 CHEMOKINE AXIS RECRUITS REGULATORY T CELLS AND MYELOID-DERIVED SUPPRESSOR CELLS IN GLIOBLASTOMA MULTIFORME

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This work is dedicated to everyone who has helped me advance in my scientific career from mentors who have taught me how to think rigorously about science to peers who have helped me persevere through the travails of graduate training. This work is also dedicated to every person with whom I have had a productive scientific discussion over the years – whether first thing in the morning or late at night, sometimes heated, and always passionate – in my mind, these conversations are the best part of science. Finally, this dissertation is dedicated to the memory of Donna Guo, a student and wonderful human being whom I had the pleasure of teaching both in the classroom and in the laboratory.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT

In many cancers, including glioblastoma multiforme (GBM), the most aggressive primary brain tumor, immunosuppression is an important aspect of tumorigenesis that manifests as the accumulation of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). However, the underlying sequence of events driving Treg and MDSC recruitment in GBM remains unknown. Here, we demonstrate that macrophages and microglia within the glioma microenvironment produce CCL2, a chemokine that recruits both $CCR4^+$ Tregs and $CCR2^+$Ly-$6C^+$ monocytic MDSCs. In murine gliomas, we established novel roles for tumor-derived CCL20 and osteoprotegerin in inducing CCL2 production from macrophages and microglia. Furthermore, tumors grown in CCL2 knockout mice fail to maximally accrue Tregs and monocytic MDSCs. We demonstrate using mixed-bone marrow chimera assays that CCR4-deficient Tregs and CCR2-deficient monocytic MDSCs are defective in glioma accumulation. Finally, targeting this axis using a small molecule CCR4 antagonist significantly improved median survival. The significance of our findings is further corroborated by GBM patient data where high CCL2 gene expression is correlated with reduced overall survival. Furthermore, we observed that both macrophages and glial cells produce CCL2 in GBM patients. Collectively, we provide novel evidence supporting a paradigm in which glioma cells influence the microenvironment to recruit effectors of potent immunosuppression.

Chapter I. Introduction

Sections of this chapter have been adapted from: Chang A.L., Miska J., Wainwright D.A., Dey M., Rivetta C.V., Yu D., Kanojia D., Pituch K.C., Qiao J., Pytel P., Han Y., Wu M., Zhang L., Horbinski C.M., Ahmed A.U., and Lesniak M.S. 2016. CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. *Cancer Research*. 76(19):5671-5682.

Overview

The link between immunity and cancer has been explored for almost a hundred years throughout the history of immunological research, from William Coley's early work with *Streptococcus pyogenes* (so-called Coley's toxins) to the development of modern immunotherapeutics of cancer vaccines, adoptive cell transfer, and checkpoint blockade. The anti-tumor capacity of T cells is one of the most potent weapons in the conflict between tumor cells and the anti-tumor immune response. Yet, like pathogens, cancers have devised a multitude of mechanisms for resisting and evading the immune response of the host. Foremost among these barriers is a potently immunosuppressive microenvironment characterized by immunosuppressive cytokines and the infiltration of cells which are capable of inhibiting a productive anti-tumor immune response. These hallmarks of immunosuppression are a feature of glioblastoma multiforme (GBM), a devastating disease that is the most common malignant brain tumor in adults. Both regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are major cellular contributors to the immunosuppressive glioma microenvironment. How these cells traffic to the brain and which signals direct the accumulation of these cells remain incompletely understood.

Cancer and immunity

From the end of the 19th century onwards, the beginning of "non-specific" immunotherapy was used experimentally by William Coley. Coley's toxins were based on *Streptococcus pyogenes*, a deadly pathogen responsible for erysipelas (Coley, 1893; Hall, 1997; Starnes, 1992). Co-infection of cancer patients was performed in an attempt to activate the immune system to clear the tumor. However, the efficacy of this approach was obscured by small sample size, inter-patient variability of *S. pyogenes* infection, and a lack of mechanistic explanation. Cellular immunity (later refined to the T cell compartment) was gradually implicated in the phenomena of tumor recognition, hypersensitivity, and transplant rejection during the mid-20th century. The fields of tumor immunity and transplantation immunity developed in parallel to each other through opposing end goals: the transplantation immunologists were attempting long-term allograft survival, while early tumor immunologists were concerned with the rejection of allograft tumors. Some of the earliest evidence that lymphocytes could be involved in the rejection of tumors came from James B. Murphy, whose extensive work included some of the following seminal findings: 1) implanting spleen or bone marrow cells near the site of allografts prevents graft growth, 2) allogeneic tumor rejection occurs with an increase in the number of lymphocytes in the blood, and 3) X-ray irradiation ablates the ability to reject tumors in mice that were normally resistant (this latter point was linked to earlier findings that lymphocyte numbers are massively reduced by X-ray irradiation) (Murphy, 1914; Murphy and Morton, 1915; Murphy and Taylor, 1918). Gross and colleagues obtained evidence that the immune system was capable of recognizing non-virally induced tumors (Gross, 1943). In these experiments, mice with chemically induced sarcomas which were resected were subsequently capable of rejecting a second sarcoma challenge (Gross, 1943). N. Avrion Mitchison further implicated cellular

immunity in the rejection of allografts and allograft tumors in his experiments demonstrated that passive transfer of immunity could only be accomplished with transfer of draining lymph nodes and not serum, peritoneal exudate, or non-draining lymph nodes (Mitchison, 1953; Mitchison, 1954; Mitchison, 1955). The recirculating small lymphocyte itself was definitively implicated as the initiator of immunological responses by Jim Gowans in experiments with purified small lymphocytes, cell labeling, and thoracic duct cannulation (Gowans et al., 1962). Furthermore, Gowans and colleagues demonstrated that small lymphocytes could either overcome tolerance, or transfer tolerance if a tolerant host was used as a donor (McGregor et al., 1967). Another major advance was gained from the work of Peter Gorer and George Snell delineating the genetics of histocompatibility followed by the widespread availability of fully inbred mouse strains which enabled the generation and study of syngeneic tumors (Gorer, 1948; Gorer and Schutze, 1938; Snell, 1948).

The characterization of the critical factors influencing immune-mediated tumor rejection stemmed from the discovery of interferon by Jean Lindenmann and Alick Isaacs along with Elizabeth Carswell and Lloyd Old's description of tumor necrosis factor, which are cytokines produced by immune cells (Carswell et al., 1975; Isaacs and Lindenmann, 1957; Isaacs et al., 1957). Both factors can possess anti-tumor activity in the context of cancer and immunity. Following the discovery of T lymphocytes, experiments utilizing "anti-theta" (anti-T cell) serum would reveal that the T cell compartment was a key factor for the cellular rejection of tumors (Rouse et al., 1972). Using a DNA library derived from a melanoma patient and the corresponding autologous cytotoxic T cell clone, Boon and colleagues identified the first human gene coding for a tumor-associated antigen (van der Bruggen et al., 1991). This gene, *MAGEA1* (melanoma antigen family A1), was expressed across many cancer types but was absent in

normal tissues, except in male germline cells and trophoblast cells (i.e., a prototypical cancer-testis antigen). The importance of T cell recognition of tumor antigens and the capacity of cytotoxic $CD8^+$ T cell to destroy tumors given a permissive environment become increasingly apparent over the following decades (Coulie et al., 2014).

The discovery of IL-2 (first named T cell growth factor) was a critical advance for immunotherapy, since cytotoxic $CD8^+$ T cells could now be expanded *ex vivo* and reinfused into patients as a specific immunotherapy (Gillis and Smith, 1977; Morgan et al., 1976; Restifo et al., 2012; Rosenberg et al., 1994). Adoptive transfer of expanded tumor-infiltrating lymphocytes following host immunodepletion results in objective response rates of ~50% in metastatic melanoma patients (Dudley et al., 2008; Morgan et al., 2006). T cell specificity towards tumor-associated antigens has also been accomplished through T cell receptor transfection or chimeric-antigen receptors. Chimeric antigen receptors (CARs) typically utilize fused single-chain antibody recognition domains (or ligand-derived domains) with intracellular T cell receptor and co-stimulatory signaling domains (Fesnak et al., 2016). Clinical trial evaluation of CAR T cells targeting CD19 revealed striking potential for prolonged complete remission in chronic lymphocytic leukemia, likely in part due to widespread tumor-associated antigen expression with cancerous cells (Porter et al., 2011). An important advance in the understanding of T cell signaling and function was the characterization of additional positive or negative signals given to $CD8^+$ T cells during an immune response, termed co-stimulation or co-inhibition, respectively. CTLA4 and the PD-1/PD-L1 axis were among the first thoroughly-investigated co-inhibitory pathways which play important roles in the negative regulation of T cell responses in the context of autoimmunity (Freeman et al., 2000; Nishimura et al., 1999; Tivol et al., 1995; Waterhouse et al., 1995). The development of antibodies capable of tuning these signals heralded a new era for

immune therapy in tumors (Dong et al., 2002; Leach et al., 1996). By releasing these "immunological checkpoints" and enhancing T cell responses, checkpoint blockade cemented itself into the modern era of immunotherapy with clinical trials that demonstrated vastly improved survival in melanoma patients (Brahmer et al., 2012; Hodi et al., 2010; Topalian et al., 2012). The unprecedented success of checkpoint blockade in melanoma catalyzed the inception of clinical trials adapting checkpoint blockade antibodies for multiple cancers. In addition to checkpoint blockade, significant advances have been made in the development of other immunotherapeutic modalities for the treatment of cancer, including adoptive T cell transfer, engineered T cells, dendritic cell vaccines, peptide vaccines, and others (Kershaw et al., 2013; Rosenberg et al., 2008). Current challenges in cancer immunotherapy include the management of therapeutic resistance mechanisms, the identification of biomarkers which distinguish patients likely to benefit from immunotherapy, the development of combinatorial therapies/novel immunotherapy targets, the identification of tumor neo-antigens as targets, and how whole-genome sequencing approaches could inform the immunotherapy of cancer (Desrichard et al., 2016; Smyth et al., 2016; Topalian et al., 2016).

The anti-tumor capacity of T cells

The adaptive immune system is divided into T cells and B cells, which protect the host from intracellular and extracellular infection in a general manner of function. T cells utilizing αβ T cell receptors (TCRs) are divided into CD4 and CD8 subsets which recognize antigen-derived peptides in the context of the major histocompatibility complex (MHC) class II or class I, respectively. CD4 "helper" T cells are thought to function as a source of cytokines and other signals which dictate the progression of downstream immune responses, whereas CD8 "cytotoxic" T cells mediate direct killing of infected cells (and tumor cells) by using the

perforin/granzyme pathway. It is thought that this ability to recognize antigens belonging to intracellular pathogens (which would be inaccessible to antibodies produced by B cells) also grants T cells the potential to recognize tumor cells. The major caveat to tumor-specific recognition has historically been whether tumor antigens are significantly different from self-antigens. The immune system repertoire must balance the ability to recognize determinants from many pathogens against the risk of developing immune responses against self. Thus, there is a need for what Paul Ehrlich termed "horror autotoxicus," a reference to the mechanisms which prevent the formation of auto-antibodies capable mediating an organism's own immune-driven destruction (Ehrlich, 1902; Silverstein, 2001). As an important corollary of the clonal selection theory posited by F. Macfarlane Burnet, the T and B cell clones that recognize self-antigens must be purged in order to prevent auto-immunity during the development of the immune repertoire (Burnet, 1959).

Cancer antigens are broadly divided into tumor-associated antigens and tumor neo-antigens (Gubin et al., 2015). A tumor-associated antigen can be shared with normal tissues but highly overexpressed by cancer cells. Alternatively, many tumor-associated antigens are expressed during embryogenesis (e.g., carcinoembryonic antigen), restricted to germline tissues (i.e., cancer-testis antigens), or in areas of relative immune privilege (the testis is also considered an immunologically-privileged site) (Scanlan et al., 2002). Tumor-associated antigens are attractive targets in cancers such as melanoma, in which damage to normal melanocytes is a relatively sustainable cost for the clearance of malignant melanoma cells. Tumor neo-antigens consist of neo-epitopes generated through oncogenic mutations or oncogenic fusions. These neo-antigens represent more specific targets that shouldn't be present in normal tissues.

Both tumor-associated antigens and tumor neo-antigens can be recognized by T cells. For

these antigens to be seen by T cells, the tumors must undergo an immunogenic cell death that allows activated dendritic cells (DCs) to take up antigens at the site of the tumor. These DCs must then traffic to lymph nodes in order to present antigen to naïve T cells. Once activated, the T cells traverse the lymphatic systems to sites of tumor, where the cytotoxic T cells can exert anti-tumor activity. The progression from cancer cell death to immune response has been termed the cancer-immunity cycle and cancers have multiple mechanisms for inhibiting each stage of the cycle (Chen and Mellman, 2013). At some point during tumorigenesis, there may be phases of outgrowth followed by immune-mediated elimination of tumor cells that is dependent on the presence of tumor antigens. This paradigm is known as "cancer immunoediting" and has been extensively studied in a methylcholanthrene-induced fibrosarcoma model (Schreiber et al., 2011; Shankaran et al., 2001). Despite the initial equilibrium phases between tumor and immune cells, cancer cells are thought to eventually shift the balance in favor of tumorigenesis through multiple mechanisms, including the loss of specific tumor antigens which can be targeted by the immune system.

The role of immunosuppression in cancer

Tumors utilize additional immunosuppressive mechanisms to promote growth and metastatic spread. Across many different cancer types, immune cell exclusion is a common barrier to the induction of anti-tumor immune responses. In the absence of the appropriate antigen-presenting cell subsets in the tumor microenvironment, downstream infiltration of cytotoxic $CD8^+$ T cells does not occur (Broz et al., 2014). The lack of immune cell infiltration in the tumor microenvironment can be attributable to specific oncogenic lesions (e.g., loss of *PTEN*), lack of specific chemokines, or physical barriers as in the case of fibrosis (Peng et al., 2016; Roberts et al., 2016; Spranger et al., 2015; Stromnes et al., 2015).

Another facet of cancer immunosuppression is the negative regulation of the effector T cell response. This can occur through the engagement of multiple co-inhibitory molecules as in immune cell checkpoints (e.g., CTLA4, PD-1/PD-L1), secretion of immunosuppressive cytokines (e.g., TGFβ, IL-10), or immunosuppressive enzyme activity (e.g., indoleamine 2,3-dioxygenase or the ectonucleotidases CD39/73). Finally, the recruitment of immunosuppressive cell subsets is another major mechanism for inhibiting productive anti-tumor immune responses. Two of the most well-established immunosuppressive cells found in cancers are Tregs and MDSCs.

Immunosuppression and immunotherapy in glioblastoma multiforme

GBM (WHO Astrocytoma Grade IV) is the most common malignant adult brain tumor with a poor prognosis (Ostrom et al., 2015). Median survival of newly diagnosed GBM patients is just 14.6 months despite the standard-of-care regimen of surgical resection, radiotherapy, and concomitant as well as adjuvant temozolomide (TMZ) (Stupp et al., 2005). High levels of immunosuppressive cytokines, Tregs, and MDSCs can all be found in the GBM microenvironment (Crane et al., 2012; El Andaloussi and Lesniak, 2007; Heimberger et al., 2008; Perng and Lim, 2015; Raychaudhuri et al., 2011). Treg infiltration is higher in GBM compared to lower grade astrocytomas, though the prognostic value of Treg infiltration for GBM patients has been controversial (El Andaloussi and Lesniak, 2007; Fecci et al., 2006; Heimberger et al., 2008; Jacobs et al., 2010; Sayour et al., 2015; Thomas et al., 2015). Increased peripheral MDSCs are found in the blood of GBM patients (Fujita et al., 2011). In addition, monocytes from healthy donors acquire MDSC characteristics when treated with conditioned media from GBM cell lines (Raychaudhuri et al., 2011). Finally, tumor-infiltrating MDSCs in GBM express high levels of PD-L1 and are capable of inducing PD-1 expression on $CD4^+$ effector memory T

cells when co-cultured (Dubinski et al., 2016). The development of immunotherapies for GBM has only recently matured to the clinical trial stage, with the vast majority of clinical trials beginning after the year 2006 (Calinescu et al., 2015). The anti-CTLA4 antibody ipilimumab is being evaluated in a Phase III clinical trial for recurrent GBM (NCT02017717), two antibodies against PD-1, durvalumab (NCT02336165) and pembrolizumab (NCT02337491), are in Phase II clinical trials for primary GBM. In addition, the anti-PD-1 antibody nivolumab is being tested in a Phase III clinical trial in recurrent GBM patients (NCT02017717) (Tivnan et al., 2016). In the Phase III trial, nivolumab is being tested alone and in combination with either bevacizumab (an anti-VEGF-A antibody) or ipilimumab. Adoptive cell transfer immunotherapies against GBM-associated antigens are also in clinical trials, with active Phase I and Phase II trials for chimeric antigen-receptor T cells targeting EGFRvIII (NCT02209376, NCT01454596), IL13Rα2 (NCT01082926), and HER2 in T cells pre-selected to recognize cytomegalovirus (NCT01109095) (Tivnan et al., 2016). Peptide-based and cellular-based vaccine immunotherapies are also currently being evaluated in clinical trials. IMA950 is a multitarget peptide vaccine against 11 antigenic targets which induced multiple tumor-associated antigen responses in at least 30% of patients in Phase I/II trials (NCT01222221, NCT01920191) (Rampling et al., 2016). Current dendritic cell vaccines for GBM in clinical trials utilize either autologous tumor lysates or target cytomegalovirus proteins (Platten et al., 2016; Tivnan et al., 2016). How these and other immunotherapy strategies can overcome the immunosuppressive GBM microenvironment while limiting immune-related adverse events is crucial information for the development of effective treatments for GBM.

The field of regulatory T cells: inception, death, and revival

The T suppressor cell (Ts) was a cellular population first described by Richard Gershon in the early 1970s as a splenocyte population that existed in tolerized animals capable of suppressing immune responses to sheep red blood cells (Gershon and Kondo, 1971). However, complete characterization of this suppressor subset was beset with many problems, including the failure to identify specific determinants which differentiated Ts from other T cell populations (and the infamous Ts specific I-J region of MHC that was never found), the difficulty in isolating a Ts clone that persisted *in vitro* with suppressor capacity, and the lack of identification of antigen-specific suppressor factors (Germain, 2008). Thus, interest in the Ts phenomenon waned over the following decades, until a revived interest in Tregs (characterized as $CD4^{+}CD25^{high}$ T cells) became a full-fledged field with the elucidation of how the transcription factor FOXP3 plays a central role in Treg function by Fred Ramsdell, Steven Ziegler, Shimon Sakaguchi, and Alexander Rudensky (Brunkow et al., 2001; Fontenot et al., 2003; Hori et al., 2003). The discovery of FOXP3 solidified the Treg population as the cell population hinted at by the previous research on Ts cells. These studies demonstrated that the transcription factor FOXP3 specifically delineates $CD4^{+}$ Tregs and is a central regulator of Treg function.

Tregs are FOXP3-expressing $CD4^{+}$ T cells with important roles in tolerance, as exemplified by the autoimmune symptoms which are present in the context of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which is a disease linked to FOXP3 dysfunction. There are two major subsets of Tregs which have been best characterized through studies in mice: thymic Tregs (tTregs) which gain Foxp3 expression in the thymus, and peripheral Tregs (pTregs) which acquire Foxp3 expression in peripheral tissues. A major question related to Treg development is how and when a T cell diverts into the Treg lineage, as well as Treg antigen specificity (Perry and Hsieh, 2016). An additional point of consideration is

the extent through which clonal deletion in the thymus and diversion into the Treg lineage act as mechanisms of central T cell tolerance. Recent papers have addressed these questions by demonstrating a role for the transcription factor Aire and using tetramer staining of model self-antigens to quantify how T cells recognizing various self-antigens are either deleted or become Tregs (Legoux et al., 2015; Malchow et al., 2016; Malhotra et al., 2016).

Tregs possess multiple potential mechanisms that likely contribute in combination to suppression of effector T cell responses (Josefowicz et al., 2012). The Treg population is characterized by high expression of CD25, the α subunit of the IL-2 receptor, which led to the hypothesis that Tregs could outcompete other T cells for limited IL-2. Early studies implicated contact-mediated suppression and the inhibition of IL-2 production as a part of Treg function in *in vitro* suppression assays (Takahashi et al., 1998; Thornton and Shevach, 1998). Tregs also express high levels of the co-inhibitory molecule CTLA4 as an additional suppressive mechanism. Other surface molecules that are highly expressed on Tregs, including GITR, LAG-3, and the ectoenzymes CD39/73 have been implicated in Treg suppressive function as well. Tregs are also capable of producing several immunosuppressive cytokines, including TGFβ and IL-10. Continuous TCR signaling is thought to be important for the peripheral function of Tregs (Levine et al., 2014), though many questions remain concerning how exactly tTregs and pTregs enforce antigen-specific immunity in the periphery as well as how antigenic specificity of Treg responses compares to that of CD4 and CD8 T cells.

Treg infiltration is observed across many different cancer types and has been correlated with poor prognosis (Liu et al., 2016). In ovarian carcinoma, the presence of Tregs correlates with poor prognosis (Curiel et al., 2004). The presence of Tregs is also a negative prognostic factor in renal cell carcinoma, hepatocellular carcinoma, and lung carcinoma (Jensen et al., 2009; Sasaki

et al., 2008; Tao et al., 2012). However, there are certain cancer types where the accumulation of intratumoral Tregs may be indicative of prolonged survival, including head and neck cancer and colorectal carcinoma (Badoual et al., 2006; Frey et al., 2010). Furthermore, additional reports have implicated Treg infiltration as both a positive and negative prognostic factor in breast and ovarian cancers. Many biological factors are likely to play a role in these observations, including the role of inflammation in driving certain cancer types, a differential role of Tregs in early versus late-stage tumorigenesis, heterogeneity of intratumoral Tregs, and the presence of other immune cell subsets in the tumor microenvironment. Treg depletion results in the potentiation of anti-tumor immune responses and prolonged survival in multiple preclinical cancer models, which has led to multiple therapeutic modalities designed to target Treg populations in patients (Liu et al., 2016). With the considerable interest in the therapeutic targeting of Tregs for cancer patients, continued advancement in understanding Treg biology is paramount for the development and refinement of immunotherapeutic strategies.

Myeloid-derived suppressor cells

MDSCs arise in the context of chronic inflammation, autoimmunity, and cancer. MDSCs are a heterogeneous population of immature myeloid cells that have gained additional immunosuppressive capacity (Gabrilovich and Nagaraj, 2009). The origin of MDSCs dates to the early 1900s with the description of two features of tumorigenesis: proliferation of hematopoietic cells outside the bone marrow and marked neutrophilia (Talmadge and Gabrilovich, 2013). Originally termed “natural suppressor” cells, the phenotype of these suppressor cells was linked to the granulocyte-monocyte colony forming cells which became abundant following cyclophosphamide induction (Brooks-Kaiser et al., 1993). The $CD11b^{+}Gr\text{-}1^{+}$ cellular phenotype was established in the late 1990s, and the term immature myeloid cell or MDSC was adopted

around this time to describe these suppressive populations (Bronte et al., 1998; Gabrilovich et al., 2001; Young et al., 1996).

$CD14^+$ monocytes capable of suppressing T cell responses were identified in the context of G-CSF/GM-CSF-induced stem cell mobilization for use in stem cell transplantation (Mielcarek et al., 1997). Further refinement of the MDSC phenotype in human patients led to a set of markers that include the common myeloid markers CD33 and CD11b, lineage-negativity ($CD3^-$ $CD19^-CD56^-CD13^-$), and low HLA-DR expression. A $CD15^+$ granulocytic MDSC population (also referred to as polymorphonuclear MDSCs or PMN-MDSCs) is a predominant MDSC population in renal cell carcinoma (Rodriguez et al., 2009).

The multiple mechanisms of MDSC-mediated immunosuppression include IL-10, inducible nitric oxide synthase (iNOS), arginase, ROS production, cyclooxygenase 2 (COX2), and acting directly as suboptimal antigen-presenting cells (Nagaraj et al., 2010; Talmadge, 2007). MDSCs express low levels of MHC class II and do not express high levels of costimulatory molecules. Thus, antigen presentation to T cells is likely to be insufficient for sustained T cell response. In addition to suppressing anti-tumor immune responses, MDSCs also promote tumor progression and metastatic spread. MDSC-derived VEGF, bFGF, and MMP9 promote tumor growth, invasion, and angiogenesis (Marvel and Gabrilovich, 2015; Pekarek et al., 1995; Yang et al., 2004; Yang et al., 2008).

Leukocyte migration signals, chemokine-chemokine receptor interactions, and immunity

During an immune response, cells must travel between target tissue sites and lymphoid organs. T cells, B cells, and DCs must also come into contact and then travel within lymph nodes. Leukocyte localization signals play a major role in directing immune cell traffic; these signals include sphingosine-1-phosphate (S1P), integrins, CD62L interactions, and chemokines.

Of all these signals, chemokines are capable of providing tissue-specific information for immune cell trafficking to anatomical sites and lymph nodes, in part controlled by the large family of chemokine/chemokine receptors (Zlotnik and Yoshie, 2012). Chemokines are divided into two large superfamilies which include C-C motif and C-X-C motif chemokines, located in separate chromosome clusters. The first chemokines were identified in the late 1980s and early 90s, followed by a second round of discoveries in the mid-1990s. Chemokines bind to subgroup A Gαi-coupled G-protein coupled receptors (GPCRs). The structural features of chemokine specificity are located at the chemokine N-terminus, which interacts extensively with the chemokine receptor, as demonstrated in two papers utilizing viral chemokine/chemokine receptor analogs to solve the structure of the chemokine-chemokine receptor complex (Burg et al., 2015; Qin et al., 2015).

As mentioned earlier, chemokines direct the homing of leukocytes to several different tissue types. These include mucosal tissues (CCL25, CCL28), the skin (CCL27, CCL17, CCL22), in the stem cell niche (CXCL12), and within lymph nodes (CCL19, CCL21). In parallel with many other aspects of immunity, the specificity of chemokine-chemokine receptor interactions must be balanced with some level of degeneracy to allow for effective leukocyte coverage of the tissues within the body. Certain chemokine receptors have evolved to recognize a few different chemokine (and some non-chemokine) ligands on a graded affinity scale. For example, CCR7 recognition of CCL19/CCL21 plays a central role in leukocyte and DC trafficking to secondary lymphoid tissues. Although both CCL19 and CCL21 are present in the T cell zone, CCL21 is exclusive to the high endothelial venules (HEV) and vessels of the afferent lymph. Conversely, CCL19-CCR7 signaling results in the internalization of CCR7 and subsequent desensitization (Schumann et al., 2010). Given these characteristics, one model is that CCL21 acts as an initial

signal that results in leukocyte trafficking to HEVs followed by subsequent signaling by CCL19. The resultant downregulation of CCR7 is important for B cells and follicular helper T cells to traffic towards the B cell zone in response to follicular DC-derived CXCL13. In this way, multiple chemokine receptor ligands play functionally distinct roles in shaping the migration of leukocytes in immunity.

A historical view of the central nervous system as a site of immunological privilege

The concept of immune privilege in the brain has been studied since the early 20th century, when studies first demonstrated that rat sarcomas could engraft into the mouse brain, but implanting the tumor cells intramuscularly or subcutaneously failed to result in xenograft tumor outgrowth (Galea et al., 2007; Shirai, 1921). Follow-up work suggested that successful engraftment of xenograft tumors in the brain required an uncoupling between the brain and immune system, as co-transplantation of autologous recipient spleen and foreign donor tumor tissue into the brain parenchyma resulted in tumor rejection (Murphy and Sturm, 1923). Here, tumor rejection also occurred if grafted tissue came into contact with cerebral ventricles. However, later evidence from Peter Medawar suggested that allograft or xenografted tissues and tumors in the brain are not entirely invisible to the immune system. Tolerance to grafts in the brain could be overcome with a second graft inserted in the skin, suggesting that the immune system could detect these graft antigens in the brain, but did not encounter the antigens in a manner that induces an immune response (Medawar, 1948). The limited capacity of the immune system to mount a response in various anatomical sites was termed "immunological privilege" by Billingham and Boswell in a paper on corneal allografts (Billingham and Boswell, 1953). This concept of immune privilege was later demonstrated in the context of bacteria, viruses, and adenoviral vectors (Byrnes et al., 1996; Matyszak and Perry, 1995; Stevenson et al., 1997).

These studies demonstrated that immune responses to antigens in the CNS were markedly different from the peripheral response and that there must be distinct protective mechanisms to limit aberrant immune responses. These physical barriers to CNS leukocyte entry and the unique facets of CNS antigen presentation create a cancer-immunity cycle unique to GBM (Figure 1).

Limiting the afferent and efferent arms of the immune response in the brain

The brain can be divided into the following compartments which differ drastically in terms of degree of immune activity and mechanisms limiting immune responses: the parenchyma proper, the ventricles containing choroid plexus and cerebrospinal fluid (CSF), and the meninges. For example, while it appears that immune responses are not generated against foreign material implanted into the parenchyma, the same material does indeed result an immune response if injected into the ventricles (Gordon et al., 1992). However, there are several mechanisms in the brain that limit both the afferent and efferent arms of the immune response. The afferent arm of immunity involves antigen processing and presentation to naïve T cells as the initial step in priming a T cell response. During an immune response in most non-CNS tissues, antigens are transported to draining lymph nodes and the spleen as a required step for the primary immune response. Here, the transport of antigens occurs either through a cellular route as antigen-coupled DCs traffic from tissue site to lymph node or through a soluble route within the lymph (Merad et al., 2013). There are two classical mechanisms of immune privilege within the CNS which are thought to limit the afferent arm: 1) lack of classical antigen presenting cells and 2) a lack of direct lymphatic drainage to lymph nodes. No cells bearing the classical DC immunophenotype have been identified in the uninflamed brain parenchyma. In addition, microglia are inefficient antigen-presenting cells in the context of the non-inflamed CNS. However, DCs exist in the leptomeninges and perivascular space, large numbers of macrophages are present in the

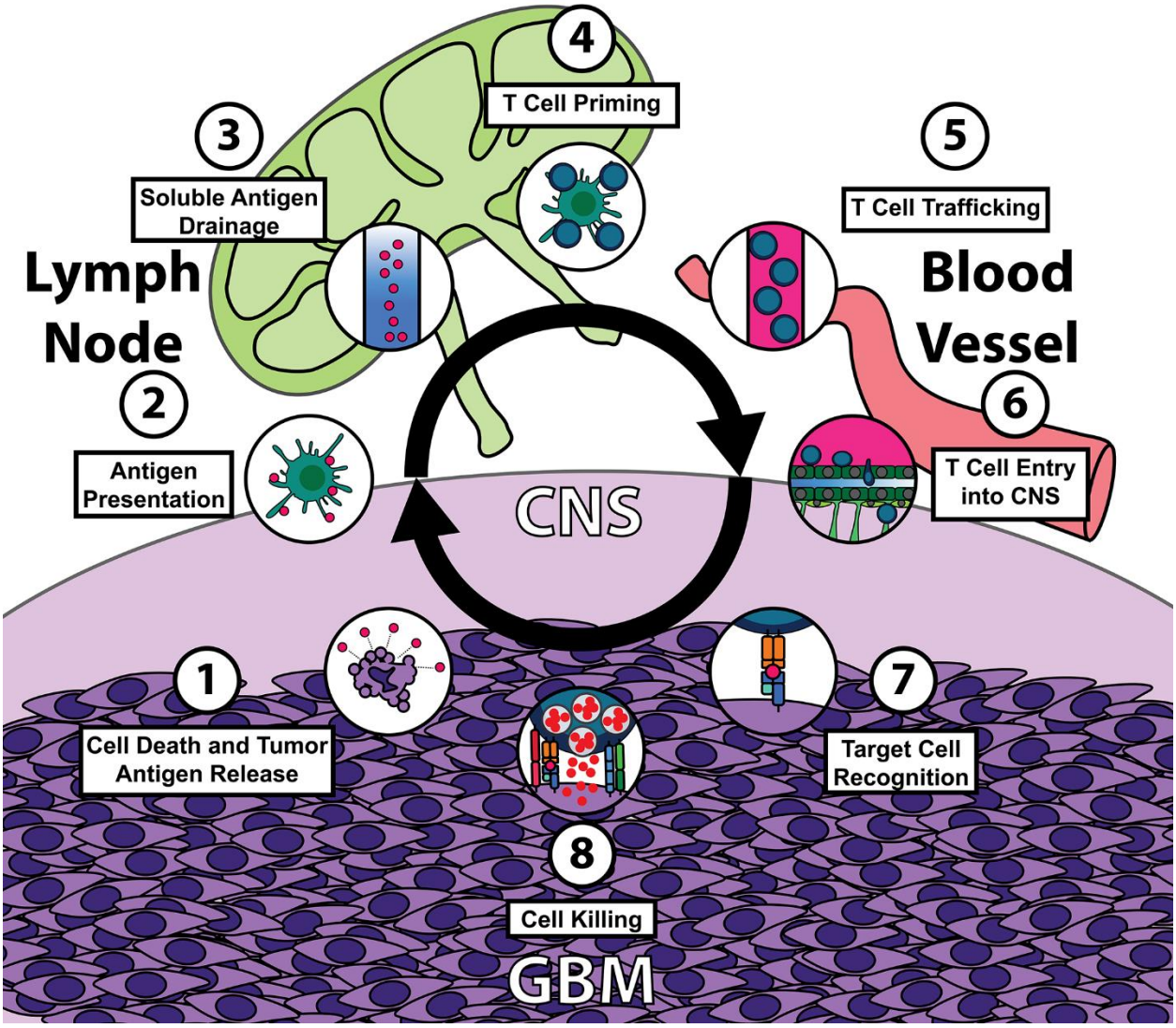

Figure 1. The cancer-immunity cycle in glioblastoma multiforme (GBM).
Upon immunogenic tumor cell death, GBM-associated antigens and neoantigens are released from dying cells (1). These antigens may be presented directly by resident antigen-presenting cells (i.e., microglia) or central nervous system (CNS) infiltrating antigen-presenting cells such as dendritic cells or macrophages (2). Alternatively, soluble tumor antigens drain into deep cervical lymph nodes through a cerebrospinal fluid route (3). There may also be a contribution of dural lymphatics (not shown) to antigen presentation and drainage in the CNS. (Note that there are only limited reports of dendritic cell migration from the CNS into lymph nodes). Following T cell priming (4), naïve T cells enter the blood stream and must cross the blood-brain barrier for entry into the CNS (5-6) (or in situ trafficking and expansion in the case of antigen presentation within tumors). Upon immune recognition of cancer cells (7), T cells mediate the killing of target tumor cells through perforin/granzyme pathways and inhibition of tumorigenesis via IFNγ and other cytokines (8), thus continuing the cancer-immunity cycle.

subarachnoid space, and the choroid plexus is populated with MHC-II expressing cells (Kolmer or epiplexus cells) which adhere to the apical aspect of the choroid plexus epithelial cells (Bartholomaus et al., 2009; Greter et al., 2005; Ling et al., 1998). Finally, the vast majority of evidence suggests that inflammatory cells carrying CNS antigens are not capable of migrating out of the CNS to a lymph node.

Although conventional lymphatic drainage is absent from the CNS, soluble antigen drainage from the brain parenchyma can occur through several routes involving cerebrospinal fluid (CSF) and interstitial fluid (ISF) that end in the cervical lymph nodes (Engelhardt et al., 2016; Louveau et al., 2015a). There is historical evidence of lymphatics in the dura mater, which has been further investigated in two recent reports demonstrating the functional capacity of dura mater lymphatic vessels to drain fluid, macromolecules, and immune cells into deep cervical lymph nodes (Aspelund et al., 2015; Louveau et al., 2015b). The flow of materials through dural lymphatics is thus potentially capable of initiating immune responses to CNS antigens.

The efferent arm of the immune response, defined as the entry and downstream activity of monocytes, T cells, and B cells into the tissue site, is also tightly regulated in the CNS. The blood-brain barrier (BBB) is a primary physical barrier for preventing the entry of leukocytes into the brain neuropil. The BBB is a functional consequence of the anatomical features of CNS capillaries which are comprised of endothelial cells, pericytes, astrocyte endfeet, and CNS-specific tight junctions (Bechmann et al., 2007). Together, this cellular structure limits both solute flow and cellular trafficking into the parenchyma. Additional mechanisms exist as a further safeguard against errant immune activation in the brain. Almost every cell in the brain expresses Fas-Ligand (Fas-L), an extracellular mediator of apoptotic cell death particularly potent against T cells (Bechmann et al., 1999). The parenchyma of the brain also possesses high

endogenous levels of immunosuppressive factors (e.g., TGFβ, IL-10) and indoleamine 2,3-dioxygenase activity (an enzyme that catabolizes tryptophan to kynurenine, inhibiting T cell proliferation) (Kwidzinski et al., 2005; Liu et al., 2006; Platten et al., 2005; Strle et al., 2001). In addition, inherent properties of neurons limit microglial immune responses. Neurotrophin secretion (e.g., from neurons) suppresses MHC class II expression in microglia (Neumann et al., 1998). In addition, the interaction between the "don't-eat-me" protein CD47 expressed on neurons and SIRPα expressed on microglia impairs microglial phagocytic capacity as well as microglial lipopolysaccharide (LPS)-induced TNFα production (Adams et al., 1998; Barclay and Van den Berg, 2014). Gangliosides, glycosphingolipids that are found abundantly in the CNS, are toxic to T cells and act on multiple stages of the effector T cell response (Irani et al., 1996). In combination, these mechanisms effectively limit leukocyte entry and attenuate efferent immune responses in the brain as another layer of control over the immune system in the CNS.

Despite the multitude of protective mechanisms against ectopic immune responses in the CNS, unchecked inflammatory responses in the brain and other CNS sites result in considerable pathology. Once CNS inflammation has been established, many of the mechanisms responsible for maintaining immune privilege are compromised. These disruptions include breakdown of the BBB, local effects mediated by pro-inflammatory cytokines and chemokines, increased antigen drainage to the periphery, the ectopic development of tertiary lymphoid tissue at the meninges, as well as the infiltration of DCs, monocytes, and macrophages (Ransohoff and Engelhardt, 2012).

Immunosuppressive cell trafficking to brain tumors

The developing glioma in the brain parenchyma represents a conflict between two opposing facets of immunological cell infiltration: 1) the relatively quiescent immunological landscape of the CNS at baseline, 2) the signals in the glioma microenvironment which promote immune cell

infiltration. The glioma microenvironment is infiltrated by highly immunosuppressive cells, frustrating anti-tumor immunity. The focus of this dissertation was to elucidate the sequence of events that results in Treg and MDSC infiltration in glioma. Importantly, a wide spectrum of immune phenotypes are observed in GBM patient tumors. Some tumors exhibit a relative paucity of immune cell infiltrate, but other tumors may be heavily infiltrated by macrophages, T cells, Tregs, and/or MDSCs. This dissertation also addresses critical pathways which differentiate GBM patients with and without immune cell infiltrate.

Chapter II. Materials and Methods

Sections of this chapter have been adapted from: Chang A.L., Miska J., Wainwright D.A., Dey M., Rivetta C.V., Yu D., Kanojia D., Pituch K.C., Qiao J., Pytel P., Han Y., Wu M., Zhang L., Horbinski C.M., Ahmed A.U., and Lesniak M.S. 2016. CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. *Cancer Research*. 76(19):5671-5682.

Cell culture. GL261 murine glioma cells (NCI Frederick National Tumor Repository Lab and mixed-cortical cell cultures (MCCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin/streptomycin solution, and Normocin (InvivoGen). Microglia were cultured in X-VIVO 15 (Lonza). Bone marrow cells, bone marrow-derived macrophages, and Tregs were cultured in RPMI Medium 1640 (Corning) supplemented with penicillin/streptomycin, L-glutamine, β-mercaptoethanol, and FBS.

Mice. All mice were bred and housed under SPF conditions in the Carlson Barrier Facility at the University of Chicago. Wild-type C57BL/6J mice (Stock 000664), *Ccl2*$^{-/-}$ mice (B6.129S4-*Ccl2*^{tm1Rol}/J, Stock 004434), *Ccr2*$^{RFP/RFP}$ mice (B6.129(Cg)-*Ccr2*$^{tm2.1Ifc}$/J , Stock 017586), and CD45.1^{+} mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ, Stock 002014), were purchased from The Jackson Laboratory. *Ccr4*$^{-/-}$ mice were kindly provided by Dr. John Belperio (University of California Los Angeles).

Patient data. Gene expression data and relevant clinical parameters were obtained from the complete GBM patient dataset available through The Cancer Genome Atlas (TCGA). IvyGAP RNA-seq and in situ hybridization data was accessed through http://glioblastoma.alleninstitute.org. Patients were stratified into CCL2-low versus CCL2-high

subsets based on CCL2 expression (< Mean - 0.5 SD and > Mean + 0.5 SD, respectively) and survival data were compared using the log rank test to determine significance. Cox proportional-hazards regression was applied to determine the role of CCL2 expression as a prognostic factor for patient survival. Finally, Pearson's correlation coefficient was used to determine the association between expression levels of CCL2 and other genes. Non-negative matrix factorization (NMF) was employed to identify clusters of CCL2 and all the other chemokines using the R package 'NMF'(Gaujoux and Seoighe, 2010). Brunet's algorithm was used to estimate the factorization. We performed 40 runs for each value of the factorization rank *r* in range 2:6 to build the consensus map. Optimal clusters were determined by the observed cophenetic correlation between clusters. Function "aheatmap" was used for plotting the heatmap and clustering with "correlation" as the distance measure and "average" as the clustering method.

Immunohistochemistry and immunofluorescence microscopy for GBM patient samples. A brain tumor tissue microarray (GL805a, US Biomax) containing 35 GBM cases was used. After xylene deparaffinization and rehydration, tissue sections were treated with antigen retrieval solution (DAKO, S1699) in a steamer for 20 minutes. Mouse monoclonal IgG1 anti-CCL2 antibody (1:100, LS-C104459, LifeSpan Biosciences) was applied to tissue sections for one hour at room temperature in a humidifying chamber followed by HRP-labeled polymer anti-mouse (DAKO, K4001), DAB+ chromogen (DAKO, K3468), and hematoxylin. CCL2 was scored on a semiquantitative basis with 0=negative, 1=weak, 2=moderate, and 3=strong. Double-immunofluorescence labeling was performed using heat-mediated antigen retrieval solution (DAKO, S1699) followed by blocking with 10% normal goat serum and CCL2 primary antibody staining with the addition of either polyclonal rabbit anti-CD163 (1:300, ab87099, Abcam) or polyclonal rabbit anti-GFAP (1:1000, Z0334, DAKO). Secondary antibody staining was

performed using goat anti-mouse IgG AlexaFluor 647 (1:1000, A-21235, ThermoFisher) or goat anti-rabbit IgG AlexaFluor 488 (1:1000, 35553, ThermoFisher) followed by a 30 minute incubation with Sudan Black and subsequent washes with 70% ethanol and then mounted using Prolong Gold Antifade mountant with DAPI (ThermoFisher, P36935). Immunohistochemistry was performed with technical support from both the Human Tissue Resource Center Core Facility at the University of Chicago and the Northwestern University Nervous System Tumor Bank. Images were captured on a Leica DMi8 inverted microscope and image analysis was with FIJI software.

Orthotopic GL261 model of GBM. 4×10^5 GL261 cells were intracranially (i.c.) implanted as previously described (Wainwright et al., 2012). Mice were anesthetized through intraperitoneal administration of a stock solution containing 100 mg/kg ketamine and 10 mg/kg xylazine. The surgical site was shaved and prepared with a swab of povidone iodine followed by a 70% ethanol. The swabbing procedure was performed three times total. An incision was then made at the midline for access to the skull. A 1-mm-diameter burr hole was drilled 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture. Mice were then placed in a stereotaxic frame and GL261 cells were injected in a total volume of 2.5 μL using a Hamilton syringe fitted with a 26-gauge blunt needle at a depth of 3 mm. The incision was then sutured using nylon thread or stapled closed. For antagonist treatment, mice were treated with the small molecule CCR4 antagonist C 021 dihydrochloride (Tocris) at 15 mg/kg-50 mg/kg doses administered subcutaneously every other day for a total of 5 doses beginning 1 day post-i.c. GL261 implantation.

Immunohistochemistry and immunofluorescence microscopy for murine brain tumors. Mice were perfused with PBS and then with 4% paraformaldehyde at 1-week post-GL261 intracranial

injection. The brains were post-fixed overnight in paraformaldehyde and then placed in a 30% sucrose solution at 4°C for 24-48 hours before cryosectioning into 20 µm sections before antibody staining. Slides were washed with TBS-T and blocked with 5% Normal Donkey Serum + 0.5% Triton X-100 (Sigma-Aldrich) PBS. Primary antibody solution: 1% BSA, PBS, 0.5% Triton X-100 (Sigma-Aldrich) overnight at 4°C. Primary antibodies: polyclonal goat anti-CCL2 (1:250, LSBiosciences), rat anti-mouse/human CD11b-biotin (1:250, Clone M1/70, BioLegend). For panels including polyclonal goat anti-mouse/rat Iba-1 (Novus Biologicals), rat anti-mouse/human CD11b (1:250, Clone M1/70, BioLegend) and hamster anti-mouse/rat/human CCL2-biotin (1:250, Clone 2H5, EBioscience) were used as primary antibodies. Secondary antibodies (2 hour incubation at 1:400 dilution): donkey anti-rabbit AlexaFluor593 (Jackson ImmunoResearch), donkey anti-goat AlexaFluor593 (Jackson ImmunoResearch) streptavidin-AlexaFluor488 (Jackson ImmunoResearch), streptavidin-AlexaFluor647 (Jackson ImmunoResearch). Mounting media used included either Fluoroshield with DAPI (Sigma-Aldrich) or Vectashield with DAPI (Vector Labs).

Images were captured with a Leica SP5 Tandem Scanner Spectral 2-photon confocal microscope (Leica Microsystems) or a Marianas Yokogawa type spinning disk confocal microscope (Intelligent Imaging Innovations). Image analysis was performed with ImageJ, FIJI, or Slidebook software. Image acquisition was performed at the University of Chicago Integrated Light Microscopy Facility.

Bone marrow-derived macrophages and cytokine treatment. Bone marrow cells were isolated from tibia, femurs, and iliac crests by flushing with complete RPMI. Cell suspension was passed through a 70 µm cell strainer and red blood cells were lysed using ACK lysis buffer (Lonza). Lysis buffer was diluted with excess RPMI, cell suspension was centrifuged, and the pellet was

resuspended in complete RPMI for cell counting. Bone marrow cells were plated in complete RPMI supplemented with 10% FBS and 10 ng/mL recombinant murine M-CSF (Peprotech). Non-adherent cells were removed after 3 days. After 5 days, bone marrow-derived macrophages were washed with PBS and treated with cytokines/chemokines or GL261 cell supernatant in RPMI + 2% FBS + M-CSF. After 24 hours of cytokine treatment, cells were washed with PBS and placed in RPMI + 2% FBS + M-CSF. Supernatant was collected after 48 hours for ELISA.

Mixed-cortical cell cultures and isolation of microglia. Performed according to a previously published protocol (Witting and Moller, 2011). Cortical plates from P3-P5 neonates were pooled, resuspended in trypsin without EDTA (Hyclone), homogenized, and washed. Neonatal mixed cortical cell cultures were used because microglia cannot be efficiently obtained from mixed cortical cell culture of older animals. Cellular preparations were then transferred to poly-ornithine (Sigma-Aldrich) coated T75 flasks. Microglia were isolated after 15-20 days *in vitro* using mechanical agitation of culture flasks and replated in 96 well round-bottom plates in X-VIVO 15 (Lonza). After 24 hours, microglia were treated with various cytokines for another 24 hour period. The supernatant was then collected after 48 hours for ELISA.

ELISA. The Ready-Set-Go! Mouse CCL2 ELISA Kit (EBioscience) was used according to manufacturer's protocol. ELISA plates (Corning Costar 9018) were coated with anti-CCL2 capture antibody overnight. The following day, plates were washed with TBS-T and blocked for 1 hour at room temperature. Fresh supernatant from macrophage or microglia cultures was added to the plate diluted 1:2 in 1× ELISA/ELISPOT diluent. Pre-diluted CCL2 standards were included at this time. Plates were incubated overnight, washed, and then incubated with anti-CCL2 detection antibody for 1 hour at room temperature. Plates were then washed and incubated

with avidin-HRP at room temperature for 30 minutes. Following at least 7 washes, plates were developed using 1× TMB substrate for ~10 minutes. The reaction was stopped using 1 M H_3PO_4.

Antibody array. Supernatant from GL261 cultures or MCCC were incubated with the Mouse Cytokine Array C6 (AAM-CYT-6, RayBiotech) and developed according to manufacturer's protocol. Film was scanned and analyzed for densitometry using ImageJ.

Flow cytometry. Tissue preparation and flow cytometric analysis was completed as previously published (Wainwright et al., 2012). Brains, cervical lymph nodes, spleens, and mesenteric lymph nodes were homogenized through a 70 µm cell strainer (Fisher). Brain leukocytes were isolated using a discontinuous 30%/70% Percoll (GE) gradient. Surface staining was completed at 37°C for improved staining of chemokine receptors. Data was acquired using an LSRII or an LSRFortessa instrument located in the Flow Cytometry Core at the University of Chicago. Flow cytometry data was analyzed using FlowJo Vx (Treestar). Fc receptors were blocked using anti-CD16/32 antibody (Clone 93, BioLegend) for 15 minutes on ice. The antibody panel for myeloid cell analysis included CD45 (Clone 30-F11, BioLegend), CD45.1 (Clone A20, BioLegend), CD45.2 (Clone 104, BioLegend), CD3 (Clone 17A2, BioLegend), CD11c (Clone N418 BioLegend), CD11b (Clone M1/70, BioLegend), F4/80 (Clone BM8, BioLegend), Ly-6C (Clone AL-21, BD Biosciences), Ly-6G (Clone 1A8, BD Biosciences), CCR2 (Clone 475301, R&D), and CCR4 (Clone 2G12, BioLegend). The antibody panel for T cell analysis included a combination of CD45.1 (Clone A20, BioLegend), CD45.2 (Clone 104, BioLegend), CD3 (Clone 17A2, BioLegend), CD4 (Clone RM4-5, BioLegend), CD8 (Clone 53-6.7, BioLegend), Foxp3 (Clone FJK-16S, EBioscience), CCR2 (Clone 475301, R&D), and CCR4 (Clone 2G12, BioLegend). All antibodies were diluted in PBS + 2% FBS + 0.01% sodium azide (Sigma-Aldrich).

RNA isolation and qRT-PCR. RNA isolation from cells was completed using the RNEasy Plus Mini Kit (Qiagen). RNA isolation from tissues was performed using IsolRNA (5Prime) and a rotor-stator homogenizer (Polytron Kinematica). Following chloroform extraction, samples were loaded onto RNeasy Plus columns (Qiagen) and processed according to the manufacturer's protocol. Total RNA was quantified using a Nanodrop apparatus (Thermo Scientific) and then converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad). Primer sequences were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) or qPrimerDepot (https://mouseprimerdepot.nci.nih.gov/) and independently validated (Cui et al., 2007; Spandidos et al., 2010). Polymerase chain reaction parameters were 1) 50°C 2 min, 1 cycle, 2) 95°C 10 min, 1 cycle, 3) 95°C 15 seconds → 60°C 30 seconds → 72°C 30 seconds, 40 cycles, 4) 72°C 10 min, 1 cycle. After the reaction was finished, samples were analyzed on a 3% agarose gel for specificity and returned to the thermocycler for dissociation curve analysis.

Primer sequences are listed below:

PBNos2 (F): 5'-GTTCTCAGCCCAACAATACAAGA-3'

PBNos2 (R): 5'-GTGGACGGGTCGATGTCAC-3'

PBArg1 (F): 5'-CTCCAAGCCAAAGTCCTTAGAG-3'

PBArg1 (R): 5'-AGGAGCTGTCATTAGGGACATC-3'

PBIL10 (F): 5'-GCTCTTACTGACTGGCATGAG-3'

PBIL10 (R): 5'-CGCAGCTCTAGGAGCATGTG-3'

PBTNF (F): 5'-CCCTCACACTCAGATCATCTTCT-3'

PBTNF (R): 5'-GCTACGACGTGGGCTACAG-3'

PBITGAM (F): 5'-GGGAGGACAAAAACTGCCTCA-3'

PBITGAM (R): 5'-ACAACTAGGATCTTCGCAGCAT-3'

PBTLR4 (F): 5’-ATGGCATGGCTTACACCACC-3’

PBTLR4 (R): 5’-GAGGCCAATTTTGTCTCCACA-3’

TGFb1 (F): 5’-CTCCCGTGGCTTCTAGTGC-3’

TGFb1 (R): 5’-GCCTTAGTTTGGACAGGATCTG-3’

IL23r (F): 5’-TTCAGATGGGCATGAATGTTTCT-3’

IL23r (R): 5’-CCAAATCCGAGCTGTTGTTCTAT-3’

IL1b (F): 5’-GAAATGCCACCTTTTGACAGTG-3’

IL1b (R): 5’-TGGATGCTCTCATCAGGACAG-3’

IL23a (F): 5’-CAGCAGCTCTCTCGGAATCTC-3’

IL23a (R): 5’-TGGATACGGGGCACATTATTTTT-3’

PD_CCL1_F 5’-ATCACCATGAAACCCACTGC-3’

PD_CCL1_R 5’-CAGCAGCTATTGGAGACCGT-3’

PB_CCL2_F 5’-TTAAAAACCTGGATCGGAACCAA-3’

PB_CCL2_R 5’-GCATTAGCTTCAGATTTACGGGT-3’

PD_CCL20_F 5’-TCTGCTCTTCCTTGCTTTGG-3’

PD_CCL20_R 5’-TGTACGAGAGGCAACAGTCG-3’

PB_CCL28_F 5’-AGAGTGAGTTCATGCAGCATC-3’

PB_CCL28_R 5’-CTGCTTCAAAGTACGATTGTGC-3’

PB_CCL22_F 5’-AGGTCCCTATGGTGCCAATGT-3’

PB_CCL22_R 5’-CGGCAGGATTTTGAGGTCCA-3’

PB_GAPDH (F): 5’-AGGTCGGTGTGAACGGATTTG-3’

PB_GAPDH (R): 5’-TGTAGACCATGTAGTTGAGGTCA-3’

Mixed-bone marrow chimera generation and competition assay. Recipient mice at least 6 weeks of age were irradiated with 1100 cGy (fractionated into two 550 cGy doses separated by 4 hours) using a Gammacell 40 Exactor irradiator (Theratronics). 24 hours post-irradiation, mice were retro-orbitally injected with a 1:1 ratio of WT CD45.1^{+} bone marrow cells or bone marrow cells from CCR4-deficient or CCR2-deficient mice resuspended in RPMI without FBS at a final volume of 150 μL. Mice were maintained on an antibiotic diet containing 275 ppm trimethoprim and 1365 ppm sulfadiazine (Uniprim, Harlan) for 14 days post-irradiation. After 6 weeks following bone marrow reconstitution, mice were injected intracranially with GL261 cells and used for experiments.

Statistical Analysis. Groups were compared with Student's two-tailed *t* test or one-way ANOVA with Tukey's test for multiple comparisons as indicated in figure legends. A p value of less than 0.05 was considered statistically significant. Survival curves were compared with the Log-rank test, corrected using the Bonferroni method if multiple survival curves were compared. All statistical tests were done using either Prism 6.0 (Graphpad) or Stata (Statacorp).

Study approval. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Chapter III. Glioma microenvironment-derived CCL2 recruits regulatory T cells and myeloid-derived suppressor cells

Sections of this chapter have been adapted from: Chang A.L., Miska J., Wainwright D.A., Dey M., Rivetta C.V., Yu D., Kanojia D., Pituch K.C., Qiao J., Pytel P., Han Y., Wu M., Zhang L., Horbinski C.M., Ahmed A.U., and Lesniak M.S. 2016. CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. *Cancer Research*. 76(19):5671-5682.

Overview

The accumulation of immunosuppressive cells is a characteristic of many different cancer types. Glioblastoma multiforme, the most common malignant brain tumor, is one cancer with a potently immunosuppressive microenvironment in which there is infiltration of regulatory T cells and myeloid-derived suppressor cells. However, mechanistic details of specific recruitment signals for these immunosuppressive cells have not been thoroughly established. In the work described in this chapter, macrophage-derived CCL2 was found to be a chemokine which drives the recruitment of monocytic myeloid-derived suppressor cells and regulatory T cells to gliomas through CCR2 and CCR4-dependent interactions respectively. CCL2 production was found to be induced by factors secreted by glioma cells, including osteoprotegerin and CCL20. These findings were supported by TCGA GBM data, where high CCL2 expression was correlated with reduced overall survival and that macrophages are a source of CCL2 in GBM patient samples.

INTRODUCTION

Immune evasion is a hallmark of tumorigenesis and a potent barrier to effective cancer therapy (Hanahan and Weinberg, 2011). In a wide spectrum of cancer types, immune evasion

manifests as the recruitment of immunosuppressive regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) to the tumor microenvironment. Tregs are a FOXP3-expressing subset of $CD4^+$ T cells which play an important role in maintaining immunological tolerance to self under normal physiological conditions (Rudensky, 2011). Treg infiltration is correlated with worse prognosis in breast, ovarian, gastric, and esophageal cancers (Bates et al., 2006; Curiel et al., 2004; Ichihara et al., 2003). MDSCs originate from monocytes that gain immunosuppressive capacity under certain pathological conditions (Gabrilovich and Nagaraj, 2009; Khaled et al., 2013). Human MDSCs are characterized by the pan-myeloid marker CD33, and include monocytic $CD14^+$ and granulocytic $CD15^+$ subsets. Murine MDSCs are defined as $CD11b^+Gr\text{-}1^+$ cells, with monocytic $Ly\text{-}6C^+$ and granulocytic $Ly\text{-}6G^+$ cellular subsets.

Glioblastoma multiforme (GBM) (WHO Astrocytoma Grade IV) is the most common malignant adult brain tumor and it has a dismal prognosis. Median survival of GBM is just 14.6 months even with the treatment standard of surgery, radiation, and chemotherapy (Stupp et al., 2005). The GBM microenvironment is characterized by high levels of immunosuppressive cytokines as well as the accumulation of Tregs and MDSCs (Crane et al., 2012; El Andaloussi and Lesniak, 2007; Heimberger et al., 2008; Perng and Lim, 2015; Raychaudhuri et al., 2011). In gliomas, Treg infiltration is higher in GBM compared to lower grade astrocytomas, though conflicting reports exist on the prognostic value of Treg infiltration (El Andaloussi and Lesniak, 2007; Fecci et al., 2006; Heimberger et al., 2008). GBM patients have increased peripheral MDSCs (Fujita et al., 2011). In addition, monocytes from healthy donors acquire MDSC characteristics when treated with conditioned media from GBM cell lines (Raychaudhuri et al., 2011). Despite the importance of these cells for the progression of GBM, the mechanistic sequence of events underlying the recruitment of these cells has yet to be elucidated.

Chemokines are specificity signals that mediate the trafficking of immune cells during development, infection, inflammation, and a vast array of other immune processes (Zlotnik and Yoshie, 2012). CCL2 is a potential candidate chemokine for Treg trafficking to glioma. Patients with GBM possess an increased percentage of circulating Tregs that express CCR4, a chemokine receptor that binds to CCL2, albeit with lower affinity than for CCL17 and CCL22 (Jordan et al., 2008; Zlotnik and Yoshie, 2012). Yet CCL2 is a more predominant chemokine in glioma than either CCL17 or CCL22. Supernatant from cultured U251 GBM cells contains soluble CCL2 and can induce Treg migration *in vitro* (Jordan et al., 2008). CCL2 has also been tied to the migration of MDSCs. CCR2, a high affinity chemokine receptor for CCL2, is found on several different myeloid cell populations, including MDSCs (Lesokhin et al., 2012). *Ccl2*$^{-/-}$ mice possess markedly reduced MDSC infiltration in gliomas transduced with plasmid DNA (Fujita et al., 2011).

Although these studies implicated a role for the CCL2-CCR4/CCR2 axis, the sequence of events from the induction of CCL2 production to the *in vivo* chemokine-chemokine receptor requirements for Tregs and MDSC recruitment remains incompletely understood. We hypothesized that CCL2 recruits both Tregs and MDSCs in GBM, thus unifying the two immunosuppressive cell subsets under one axis. We sought to determine the clinical relevance of CCL2 in large scale patient data, identify sources of CCL2, and elucidate the underlying mechanisms driving Treg and MDSC accumulation. Here, we determined that CCL2 expression is a prognostic factor for patients with GBM and can be produced by both macrophages as well as glial cells in GBM patient samples. We observed that cells expressing the macrophage marker CD163 contribute to CCL2 production in GBM patients. In the GL261 immunocompetent murine model of GBM, we found that tumor-associated macrophages and microglia are major

sources CCL2 which subsequently recruits CCR4-expressing Tregs and CCR2-expressing Ly-6C$^+$ monocytic MDSCs. We established novel roles for tumor-derived CCL20 and osteoprotegerin in inducing CCL2 production from macrophages and microglia. Through mixed-bone marrow chimera studies, we observed a role for CCR4 in Treg recruitment and a requirement for CCR2 in the accumulation of monocytic MDSCs in glioma. Finally, using a small molecule antagonist, we demonstrated that the CCL2-CCR4/2 axis is a relevant therapeutic target in GBM. Collectively, these studies delineate how microenvironment-derived CCL2 results in the accumulation of Tregs and MDSCs in glioma.

RESULTS

CCL2 is a clinically relevant prognostic factor in GBM patients. To determine the clinical relevance of CCL2, we obtained gene expression data and clinical parameters from GBM patient data available through The Cancer Genome Atlas (TCGA). Patients were stratified into CCL2-high and CCL2-low groups based on CCL2 gene expression. Median survival was significantly increased in CCL2-low subset patients when compared to the CCL2-high subset in data encompassing all glioma grades (Figure 2A) as well as in data limited to GBM cases alone (Figure 2B) (Affymetrix U133A: CCL2-low 479 days, CCL2-high 375 days, $p < 0.001$) (Illumina HiSeq: CCL2-low 485 days, CCL2-high 317 days, $p = 0.003$). CCL2 expression was confirmed as a prognostic factor in GBM patients through both univariate and multivariate Cox regression analysis (HR=1.11). In contrast to CCL2, gene expression levels of CCL1, CCL3, CCL4, and CCL28 did not segregate GBM patient survival (Figure 3A-3D). To survey CCL2 protein levels in GBM, we performed immunohistochemical staining of CCL2 on a GBM patient tissue microarray with 35 GBM cases with 2 cores from each case (Figure 3H-3I). In non-tumor brain biopsies, CCL2 expression was scored as 0 (Figure 4A, left). In contrast, among the 35

GBM cases available on the tissue microarray, 16 cases were scored as 0 (no CCL2 staining), 6 cases were scored as 1 (low), 9 cases were scored as 2 (medium), and 4 cases were scored as 3 (highest staining). CCL2 expression was also correlated to the expression of the human MDSC markers CD33 ($r = 0.56$, $p < 0.001$) and CD14 ($r = 0.70$, $p < 0.01$), as well as the expression of CD4 ($r = 0.56$, $p < 0.001$) (Figure 4B-4D). Importantly, CCL2 expression was not correlated to the expression of the pan-T cell marker CD3, implying some specificity to the correlation with CD4 and MDSC markers (Figure 4E). Overall, the results suggest that CCL2 mRNA and protein expression serves as an important prognostic factor for survival in GBM.

CCL2 is present in the perinecrotic regions of GBM patient tumors. Next, we examined the subanatomical location of CCL2 expression in GBM patient tumors. We utilized RNA-Seq and in situ hybridization (ISH) data sets available through the Ivy glioblastoma atlas project (IvyGAP) (available from http://glioblastoma.alleninstitute.org/) (Allen Institute for Brain Science, 2016). In this data set, RNA-seq data was obtained by laser capture microdissection of several subanatomical locations of GBM patient samples which were annotated as cellular tumor, hyperplastic vessels, infiltrating tumor, leading edge, microvascular proliferation, perinecrotic zone, or pseudopalisading cells. The IvyGAP performed semi-automated annotation using statistical machine learning algorithms in 12,000 H&E images with confirmation and manual correction consultation by board-certified neuropathologists. We obtained RNA-Seq data for all C-C and C-X-C chemokines for clustering using non-negative matrix factorization. This clustering analysis segregated CCL2 along with other chemokines that were highly expressed in the perinecrotic zone of GBM (Figure 5A-5C). We confirmed this data through ISH data for CCL2 (Figure 6). Interestingly, ISH data for the macrophage/microglia marker CD163 also found gene expression in cells localized to perinecrotic anatomical locations (Figure 7). Thus,

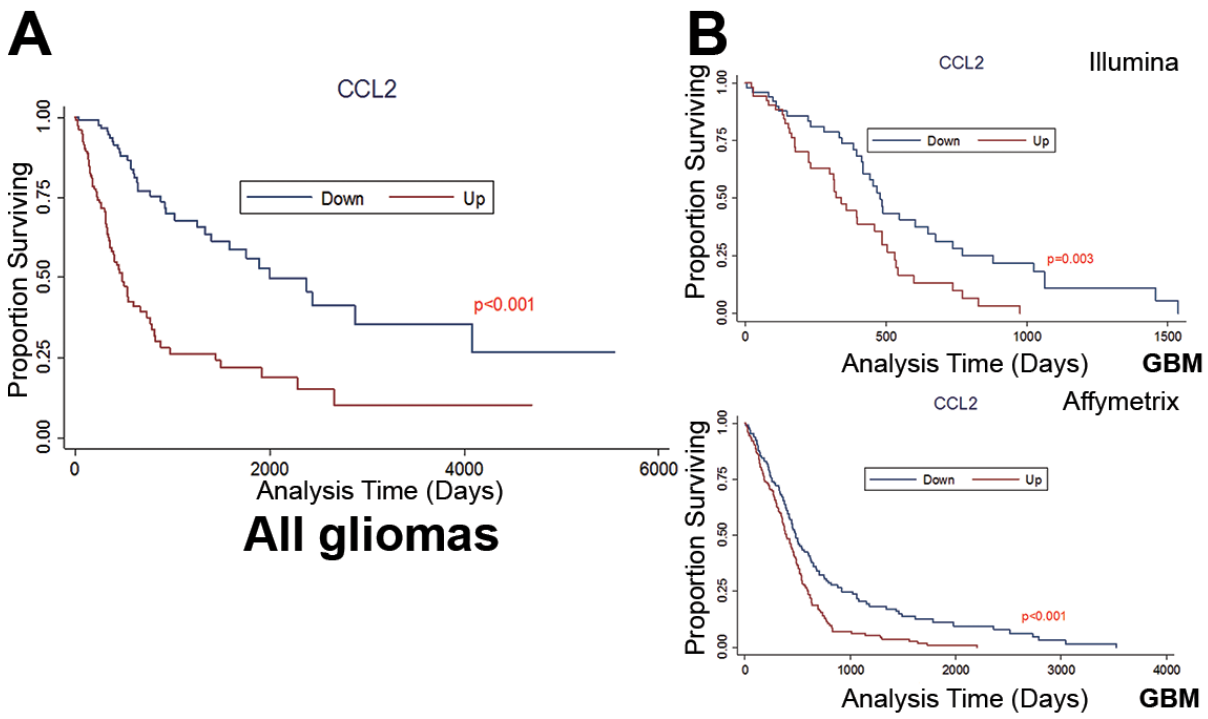

Figure 2. Low CCL2 expression is linked to improved survival in GBM.
(A) Kaplan-Meier survival curves of glioma patients (Grade I-IV) were generated from TCGA Illumina HiSeq data where patients were segregated into CCL2-low and CCL2-high expressing groups on the basis of <mean - 0.5SD (for CCL2-down) and >mean + 0.5SD (CCL2-up) (CCL2-down, *n* = 161; CCL2-up, *n* = 159). (B) Analysis restricted to GBM (Grade IV) patients for both Illumina HiSeq (CCL2-down, *n* = 42; CCL2-up, *n* = 41) (top) and Affymetrix U133A (CCL2-down, *n* = 135; CCL2-up, *n* = 134) (bottom) arrays. GBM, glioblastoma mutiforme. TCGA, the Cancer Genome Atlas. Kaplan-Meier curves were compared using the Log-rank test.

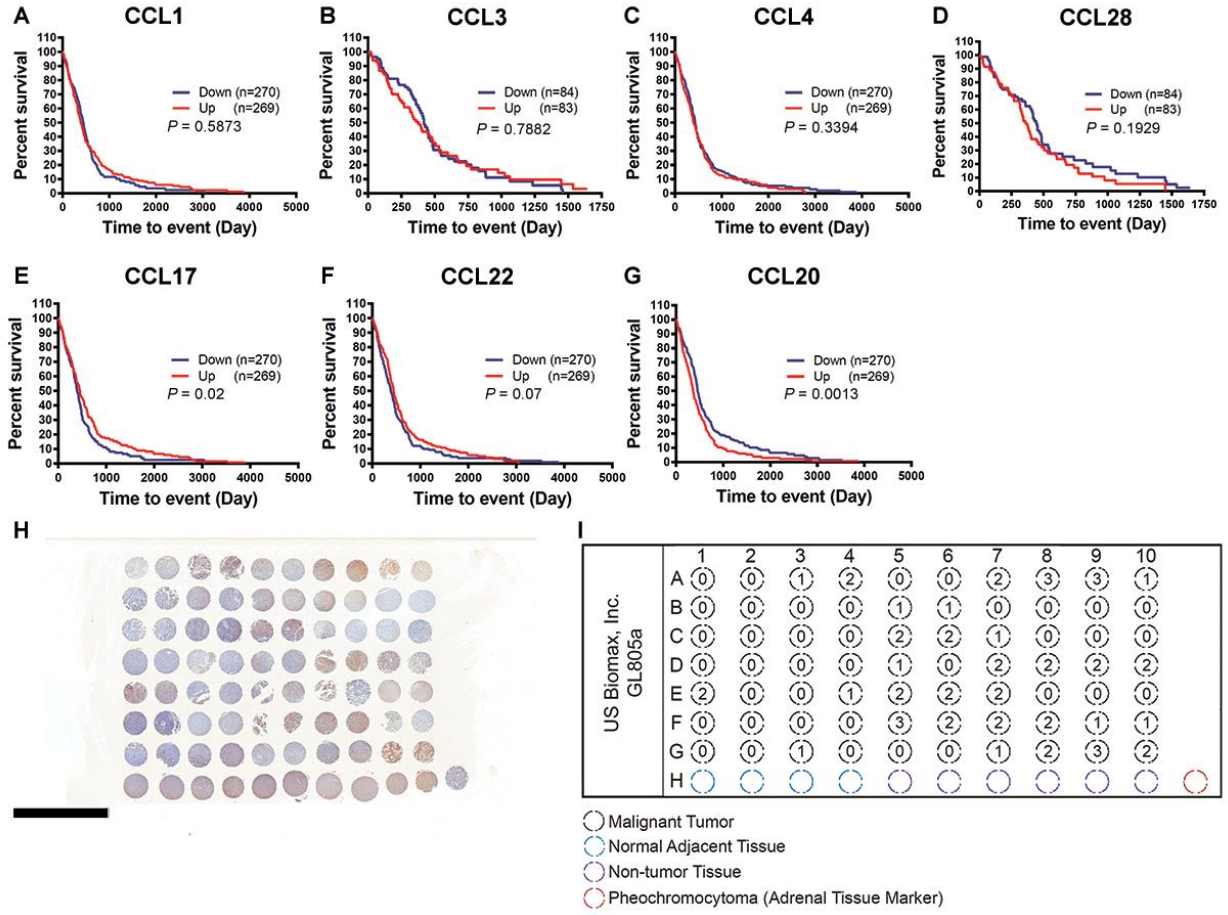

Figure 3. Clinical relevance of CCL2 in GBM compared to other candidate chemokines. (A-G) Kaplan-Meier survival curves generated from TCGA data using GBM patients segregated by gene expression for a panel of putative Treg and MDSC recruiting chemokines. (H) GBM patient tissue array stained for CCL2. (I) Key showing CCL2 score corresponding to GBM patient cores. Scale bar = 7 mm. TCGA, The Cancer Genome Atlas. Survival curves were compared with the Log-rank test

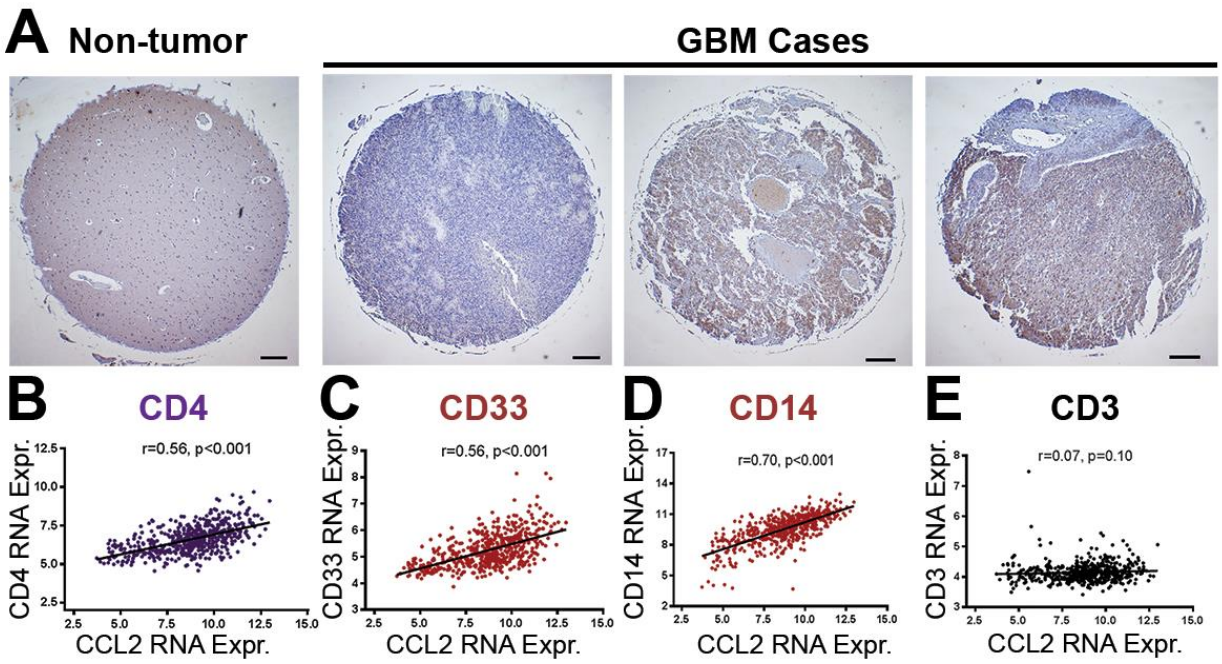

Figure 4. CCL2 is present on a protein level and CCL2 expression is correlated with gene expression indicative of immunosuppressive cell infiltration in GBM.
(A) Representative images from GBM tissue microarray. Pathology scoring from left to right: 0, 0, 2, 2 (scale of 0-3). Scale bar = 200 μm. (B-E) Pearson correlation between gene expression from GBM patient data of CCL2 expression and CD4, CD33, CD14, and CD3. GBM, glioblastoma mutiforme. TCGA, the Cancer Genome Atlas. RNA Expr., relative mRNA expression.

CCL2 expression is a characteristic feature the perinecrotic zone of GBM, an area also populated with infiltrating inflammatory macrophages, as suggested by the CD163 ISH data.

CD163-positive macrophages contribute to CCL2 production in GBM patients. Previous reports have found that CCL2 is produced by astrocytes in the context of neuroinflammation and by tumor cells in the context of glioma (Carrillo-de Sauvage et al., 2012; Jordan et al., 2008; Ransohoff et al., 1993). Because CCL2 can also be produced by tumor-associated macrophages, we determined the contributions of glial cells compared to macrophages and microglia for CCL2 production in GBM patients on a protein level (Heimdal et al., 2001; Leung et al., 1997). We performed double-immunofluorescence labeling of CCL2 and the macrophage/microglia marker CD163 in formalin-fixed, paraffin-embedded sections from 20 GBM patients. We opted for whole sections to ensure extensive anatomical coverage of GBM tumor areas. We observed heavy infiltration of CD163+ cells that were morphologically infiltrating macrophages in 13/20 GBM cases. CCL2 staining was found in both cellular tumor areas as well as in perinecrotic zones. We observed CCL2 immunoreactivity in CD163+ (Figure 8, GBM case 256) as well as in both CD163+ and CD163- cells (Figure 8, GBM case 376A1 and 257). The CD163- cells that produce CCL2 are most likely tumor cells, based on size/morphology and previous reports. Therefore, both tumor cells and glioma-associated macrophages are capable of producing CCL2 in GBM.

CCL2 is a major microenvironment-derived candidate chemokine for recruiting Tregs and MDSCs in the GL261 model of GBM. We next investigated the GL261 murine model of GBM to determine the role of CCL2 directly. Tregs and MDSCs accumulate in the brain after syngeneic GL261 astrocytoma cells are implanted into C57BL/6 mice (Wainwright et al., 2012). We determined CCL2 transcript expression localization in GL261-bearing brains at 1 week post-

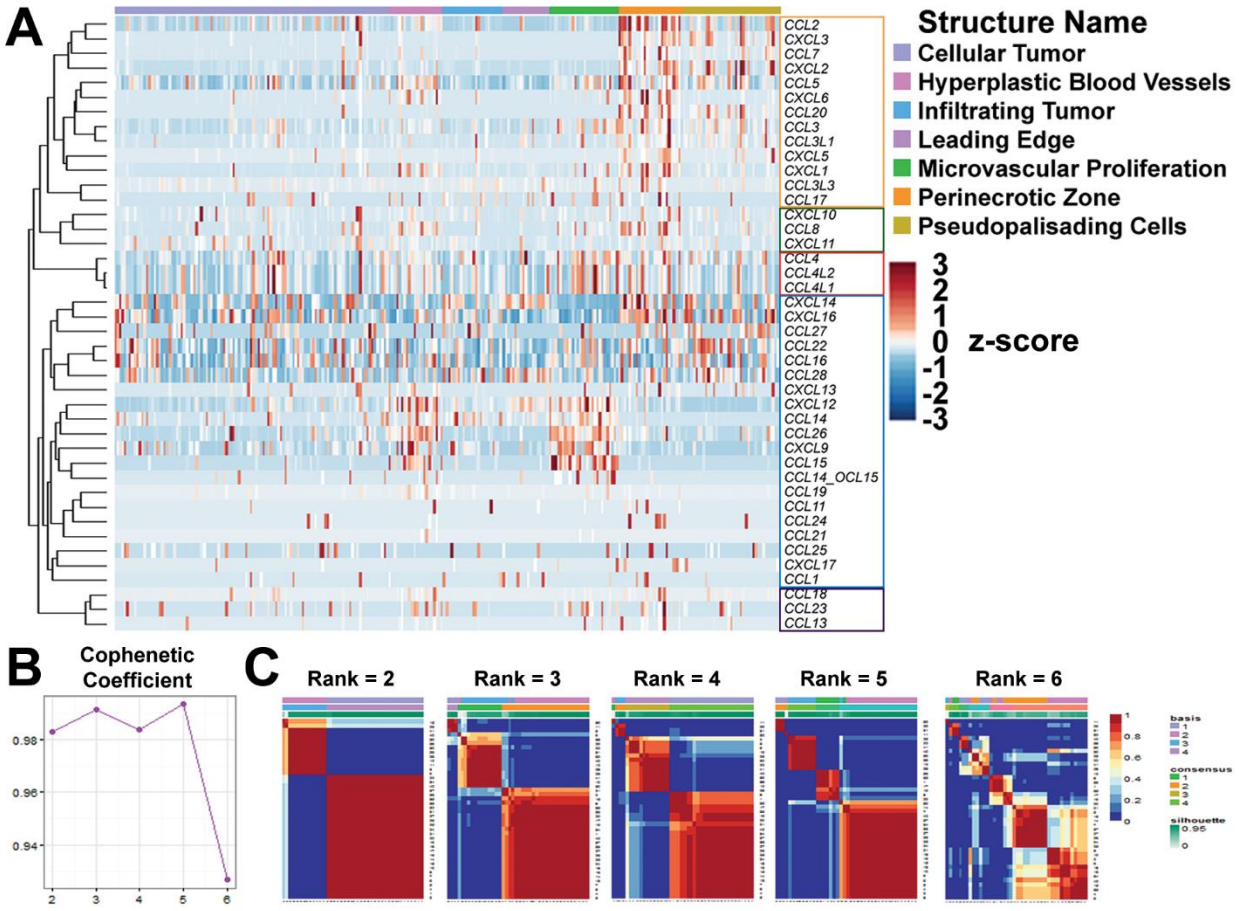

Figure 5. CCL2 is a member of a chemokine cluster that is highly expressed in perinecrotic regions of GBM.
(A) Non-negative matrix factorization clustering of IvyGAP RNA-seq data encompassing C-C and C-X-C chemokines expressed in GBM along with anatomical structure localization. Identified clusters are denoted with colored boxes around gene names. (B) Cophenetic coefficient values (y axis) for the non-negative matrix factorization process for each factorization rank of *r* (x axis). (C) Consensus map representing clustering efficacy for each factorization rank *r*. Data represent the results of 40 runs for each value of *r*. IvyGAP, Ivy Glioblastoma Atlas Project.

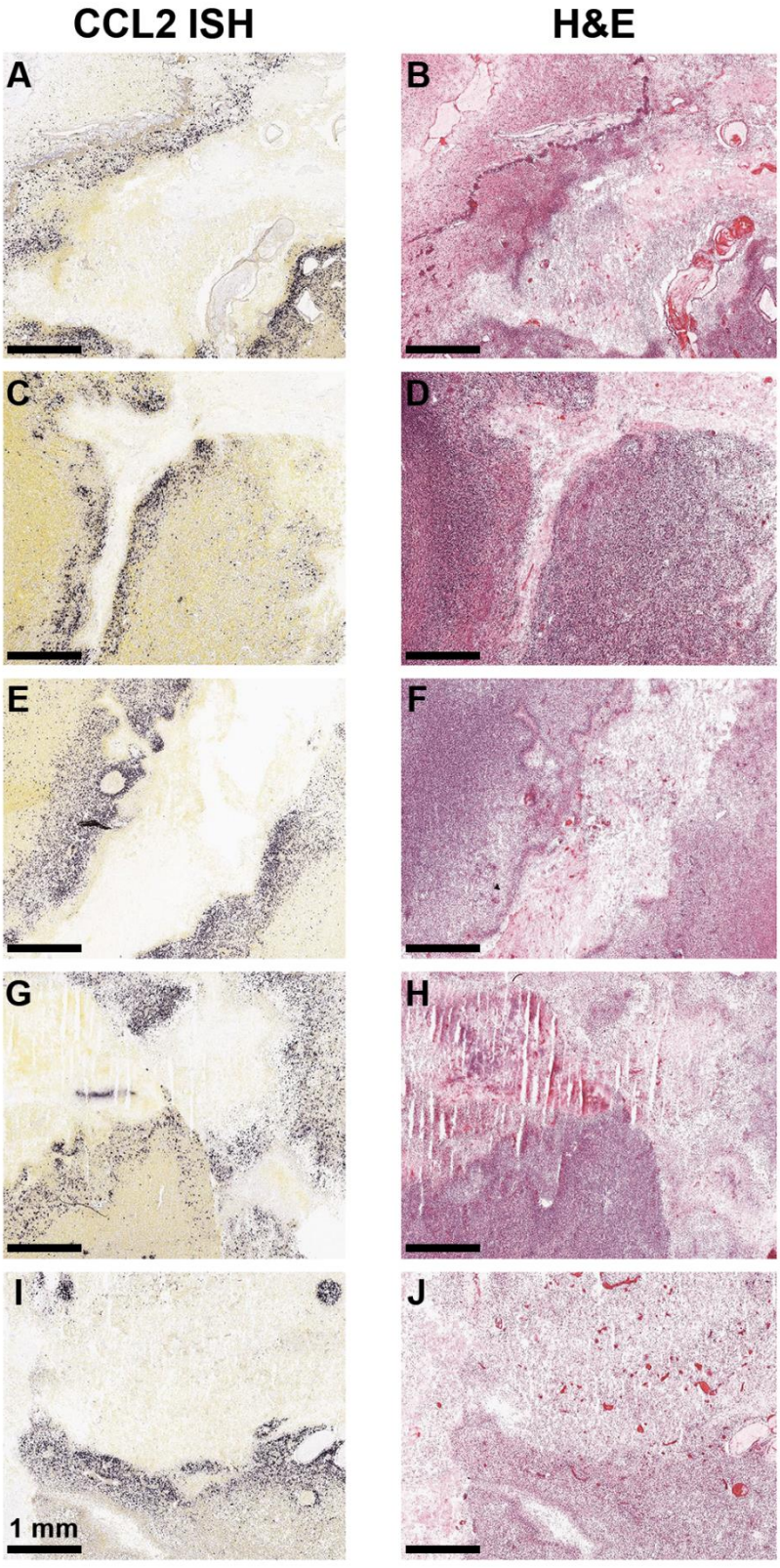

Figure 6. CCL2-expressing cells are localized to perinecrotic regions of GBM.

Figure 6, continued.
(A, C, E, G, I) *In situ* hybridization data for CCL2 in five representative GBM cases. (B, D, F, H, J) Corresponding hematoxylin and eosin staining. Images were obtained through the Ivy Glioblastoma Atlas Project web portal (Allen Institute for Brain Science).

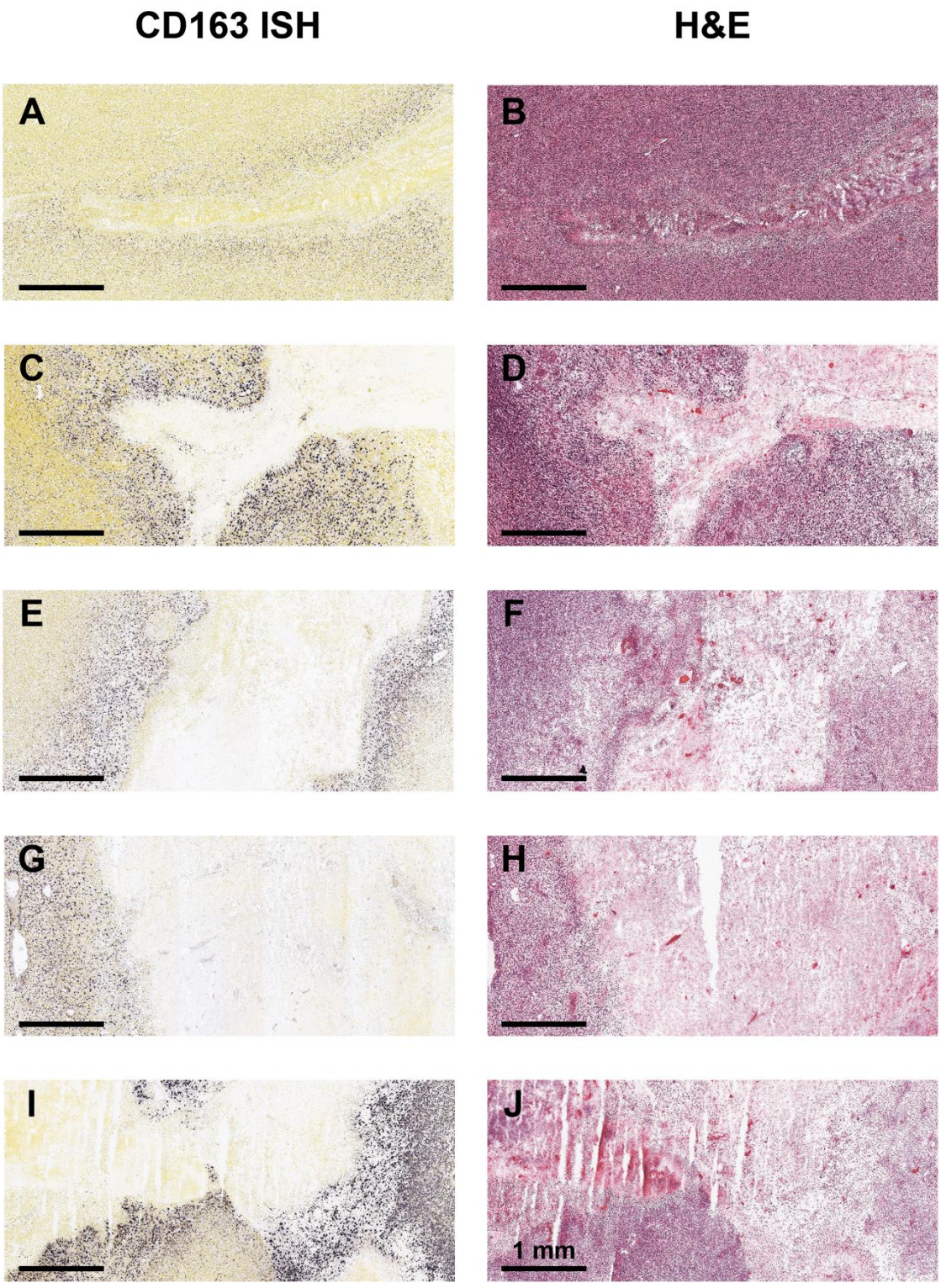

Figure 7. CD163-expressing cells populate perinecrotic zones of GBM.

Figure 7, continued.
(A, C, E, G, I) *In situ* hybridization data for CD163 in five representative GBM cases. (B, D, F, H, J) Corresponding hematoxylin and eosin staining. Images were obtained through the Ivy Glioblastoma Atlas Project web portal (Allen Institute for Brain Science).

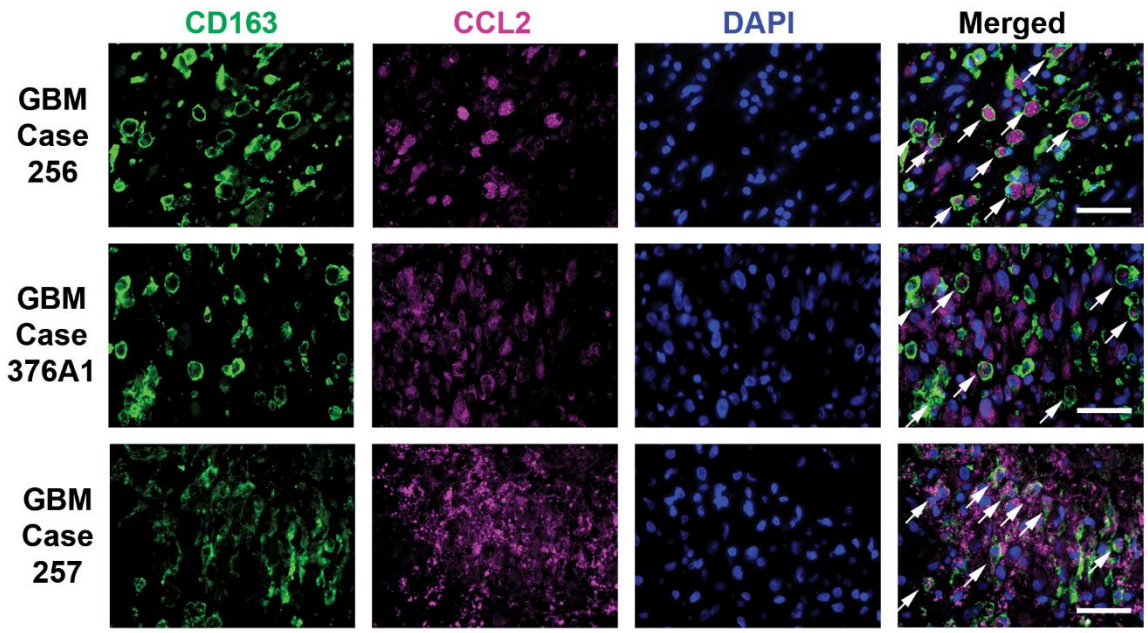

Figure 8. CD163+ macrophages are a source of CCL2 in GBM.
Double-immunofluorescence labeling of CD163 and CCL2 in three GBM cases. 40× objective. Scale bar = 50 μm. Arrows, CCL2-positive, CD163-positive cells.

injection, during the recruitment phase for Tregs and MDSCs. Strikingly, high CCL2 transcript levels were found within leukocytes isolated by density gradient centrifugation, ~20 fold over PBS control in the whole leukocyte preparation compared to 5-fold over PBS control in non-leukocytes (Figure 9A). CCL2 was expressed at the highest level compared to other candidate Treg and MDSC-recruiting chemokines (Figure 9B). CCL2 was also detected on the protein level via ELISA (260 ± 33 ng/mL in the GL261-injected hemisphere compared to 93 ± 7 ng/mL in the non-GL261-injected hemisphere, $p = 0.0075$, mean ± SEM) (Figure 9C). Collectively, these data indicate that CCL2 is present both at the mRNA and protein level in murine brain tumors and that a major source of CCL2 lies within leukocytes in the glioma microenvironment.

CD11b+ macrophages and microglia are the primary source of CCL2 in the GL261 model. To identify the cellular source of CCL2 in the GL261 model, immunofluorescence staining of tissue sections was performed at 1-week post-i.c. implantation of GL261 cells (Figure 10A). CCL2 immunoreactivity was detected in the vicinity of $CD11b^+$ and $Iba\text{-}1^+$ cells only in the context of GL261 brain tumors, but not in the brains of naïve mice. We performed intracellular cytokine staining and found that CCL2 expression was predominantly confined to $CD45^{int}CD11b^+$ microglia (~60% CCL2-positive) and $CD45^{high}CD11b^+Ly\text{-}6G^-$ macrophages (~70% CCL2-positive) (Figure 10B). In contrast, a low percentage of $CD4^+$ T cells and $CD11b^+Ly\text{-}6G^+$ granulocytic MDSCs expressed CCL2 (~10% for both $CD4^+$ T cells and granulocytic MDSCs) (Figure 10B). Given that CCL2 can be produced by myeloid cells stimulated with various polarizing conditions, we next determined the M1 or M2 status of glioma-associated microglia and macrophages in our model to identify candidate cytokines responsible for inducing CCL2 production in these cells (Cai et al., 2015). Microglia from GL261 tumors demonstrated an increased expression of the M2-associated gene Arginase-1

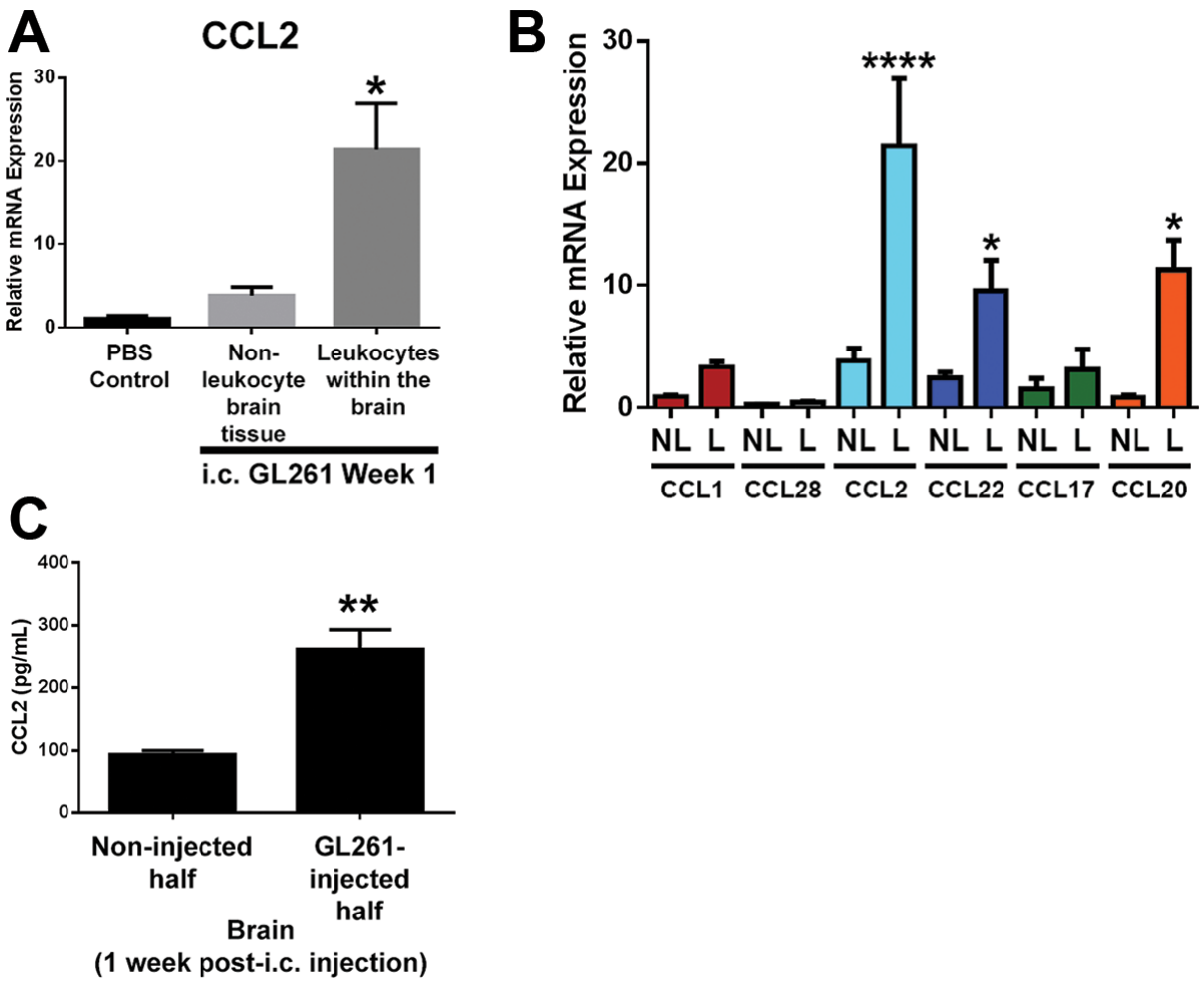

Figure 9. CCL2 mRNA and protein is detectable in the GL261 model of GBM.
(A) GL261 cells were orthotopically implanted into the right hemisphere of syngeneic immunocompetent C57BL/6 mice. At 1 week post-intracranial (i.c.) injection whole brains were homogenized followed by Percoll gradient separation of leukocytes and non-leukocytes. qRT-PCR was performed for CCL2 in mice intracranially-injected with GL261 cells (n = 4) or PBS-injected controls (n = 3). (B) qRT-PCR for CCL1, CCL28, CCL2, CCL22, CCL17, and CCL20 from non-leukocytes (NL) and leukocytes (L) as in (A). (C) Detection of CCL2 protein in both brain hemispheres at 1 week-i.c. GL261 (n = 3). Data are representative of at least 2 independent experiments. Data are represented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test (A, B) or Student's t test (C).

(*Arg1*) relative to microglia from PBS-injected control brains (Figure 10C). When compared to peripheral macrophages, glioma-associated macrophages express a ~600 fold greater *Arg1* mRNA level while glioma-associated microglia express a 60-fold greater *Arg1* mRNA level (Figure 10D). Thus, the major sources of CCL2 in GL261 gliomas are *Arg1*-expressing glioma-associated macrophages and microglia.

A soluble GL261-derived factor induces CCL2 production from macrophages and microglia. Since tumor-associated macrophages and microglia are major sources of CCL2, we hypothesized that GL261-derived factors induce CCL2 production from these cells. To test this hypothesis, we treated bone marrow-derived macrophages (BMDMs) and microglia isolated from neonatal mixed-cortical cell cultures (MCCC) with conditioned media from GL261 cells. GL261-conditioned media induced CCL2 production from both macrophages (1881 ± 40 ng/mL with conditioned media vs. 520 ± 23 ng/mL with control, $p < 0.0001$, mean ± SEM) and microglia (101.3 ± 5.1 ng/mL with conditioned media vs. 56.9 ± 0.1 ng/mL with control, $p < 0.01$) as determined by ELISA (Figure 11A-11B). Cytokine stimuli also impacted CCL2 production. LPS + IFNγ induced CCL2 production from BMDMs and microglia whereas TGFβ or TNFα decreased CCL2 production from BMDMs (42.8 ±3.9 ng/mL in TGFβ-treated BMDMs and 120.3 ± 15.7 ng/mL in TNFα-treated BMDMs, $P < 0.001$, mean ± SEM) (Figure 11A-11B). Collectively, these data suggest that soluble factors derived from GL261 cells induce CCL2 secretion from macrophages and microglia. We performed antibody arrays using GL261 supernatant and MCCC supernatant to selectively identify tumor microenvironment-specific soluble factors (Figure 12A-12B and Table 1). Using this approach, we identified the presence of osteoprotegerin (OPG), osteopontin (OPN), soluble TNF receptor type II (sTNFRII), CCL5, CCL20, and CXCL2. Among these candidate cytokines, only OPG and CCL20 induced CCL2

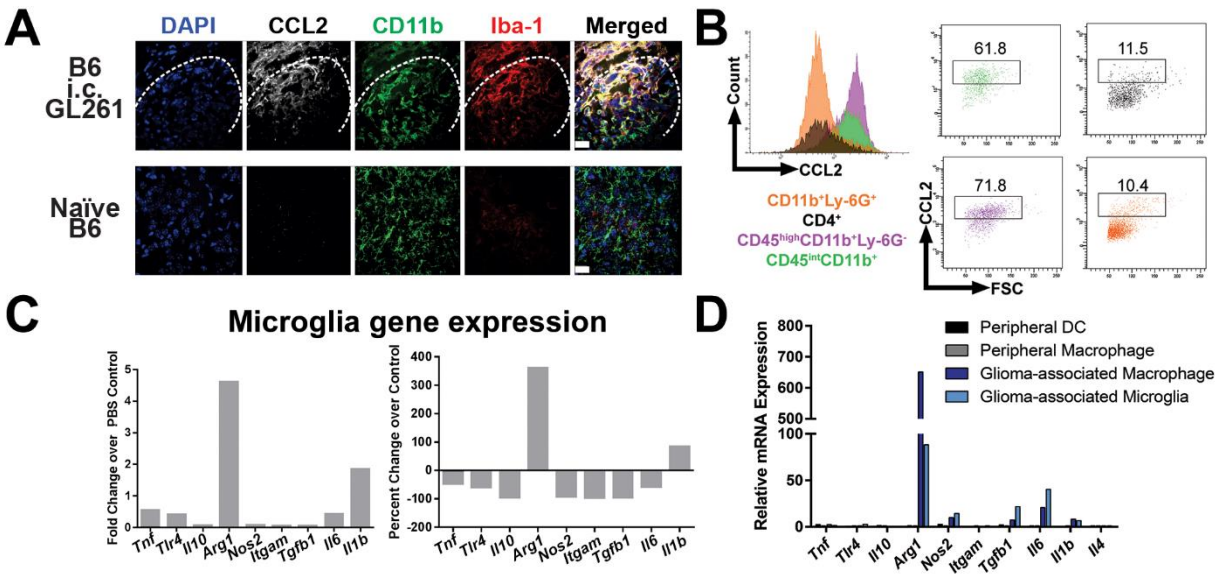

Figure 10. Myeloid cells are major sources of CCL2 in GL261 tumors.
(A) Sections of 1 week post-GL261 implanted or control brains stained for CCL2, CD11b, and Iba1. Dashed line indicates tumor border. Scale bar = 10 μm. (B) Intracellular cytokine staining of CCL2 in $CD45^{high}CD11b^{+}Ly\text{-}6G^{-}$ macrophages, $CD45^{int}CD11b^{+}$ microglia, $CD45^{high}CD11b^{+}Ly\text{-}6G^{+}$ granulocytic MDSCs, and CD4 T cells. (C) $CD45^{int}CD11b^{+}$ microglia were sorted from tumor-bearing mice (n = 5) and gene expression was analyzed by qRT-PCR compared to PBS-injected mice. (D) Expression of M1- and M2-associated genes in glioma-associated microglia and macrophages compared to peripheral macrophages and $CD45^{high}CD11b^{-}CD11c^{+}$ dendritic cells (DCs) isolated from spleens within the same mice. Images and plots in (A-B) are representative of 3 independent replicates. Data are representative of at least 2 independent experiments.

production *in vitro* (Figure 12C-12E). These data suggest that OPG and CCL20 secreted by glioma cells induce CCL2 expression in microglia and macrophages.

GL261 tumors are infiltrated by CCR4+ Tregs and CCR2+ MDSCs. We next determined which cells in the glioma microenvironment express chemokine receptors recognizing CCL2. Flow cytometric analysis of GL261-bearing brains at 1 week post-tumor implantation found that CCR4 was expressed by 60% $CD3^+CD4^+Foxp3^+$ Tregs, while CCR2 was localized to $CD3^-CD45^+CD11c^-CD11b^+$ MDSCs (Figure 13A and Figure 15A-B). In contrast, $CD8^+$ T cells did not express high percentages of either CCR4 or CCR2 (Figure 13A-13B). The highest percentages of $CCR4^+$ Tregs were observed in the brain compared to cervical lymph nodes (cLNs) or the spleen, suggesting that the accumulation of $CCR4^+$ Tregs is specific to brain tumors (Figure 13C-13D). Both Ly-$6G^+$ granulocytic and Ly-$6C^+$ monocytic MDSCs accumulated in the brain at 1 week post-i.c. GL261 implantation and are virtually absent in PBS-injected controls (Figure 14A-14B). Furthermore, both tumor-infiltrating MDSC subsets expressed CCR2 (Figure 14C-14D). In contrast, $CD45^{int}CD11b^+$ microglia expressed minimal CCR2 or CCR4 (Figure 14C-14D). In total, these results suggest that both Treg and MDSC populations possess chemokine receptors required for responding to CCL2 in the glioma microenvironment.

Tregs and Ly-6C+ monocytic MDSCs fail to maximally accumulate in the glioma microenvironment in the absence of CCL2. To delineate the requirement for CCL2 for the trafficking of Tregs and MDSCs to the tumor microenvironment, we implanted GL261 cells into *Ccl2*$^{-/-}$ mice and analyzed the brain tumor infiltrate at 1 week post-implantation. In the absence of CCL2, fewer Tregs (wild-type B6 ~1.52×10^4, *Ccl2*$^{-/-}$ ~6.46×10^3, $p = 0.0260$, mean absolute numbers of $CD3^+CD4^+Foxp3^+$ Tregs) and monocytic Ly-$6C^+$ MDSCs (wild-type B6 ~7.21×10^4,

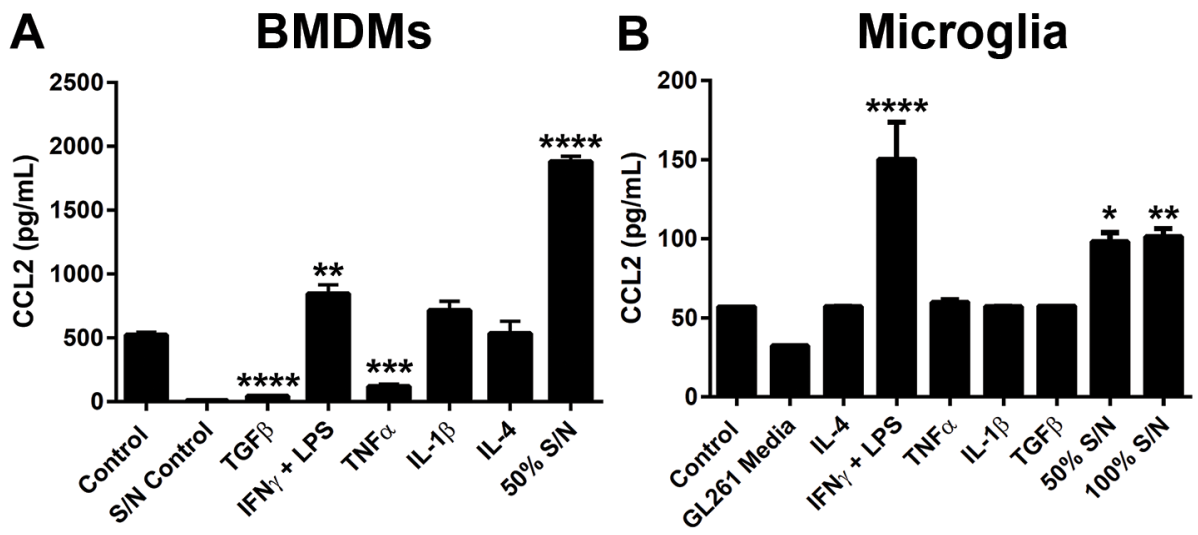

Figure 11. Soluble GL261 factors induce CCL2 production from macrophages and microglia.

(A) Bone marrow-derived macrophages (BMDMs) were treated for 24 hours with cytokines or media containing 50% GL261 cell supernatant. Supernatant was collected after another 48 hours for CCL2 analysis by ELISA. Supernatant (S/N) control denotes the CCL2 content in GL261 supernatant. (n = 3-6). (B) Microglia from neonatal mixed-cortical cell cultures (MCCC) were treated with the indicated cytokines or with media containing GL261 supernatant. (n = 3). Data are representative of 3-4 independent experiments. Data are represented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ by ordinary one-way ANOVA with Tukey's multiple comparisons test.

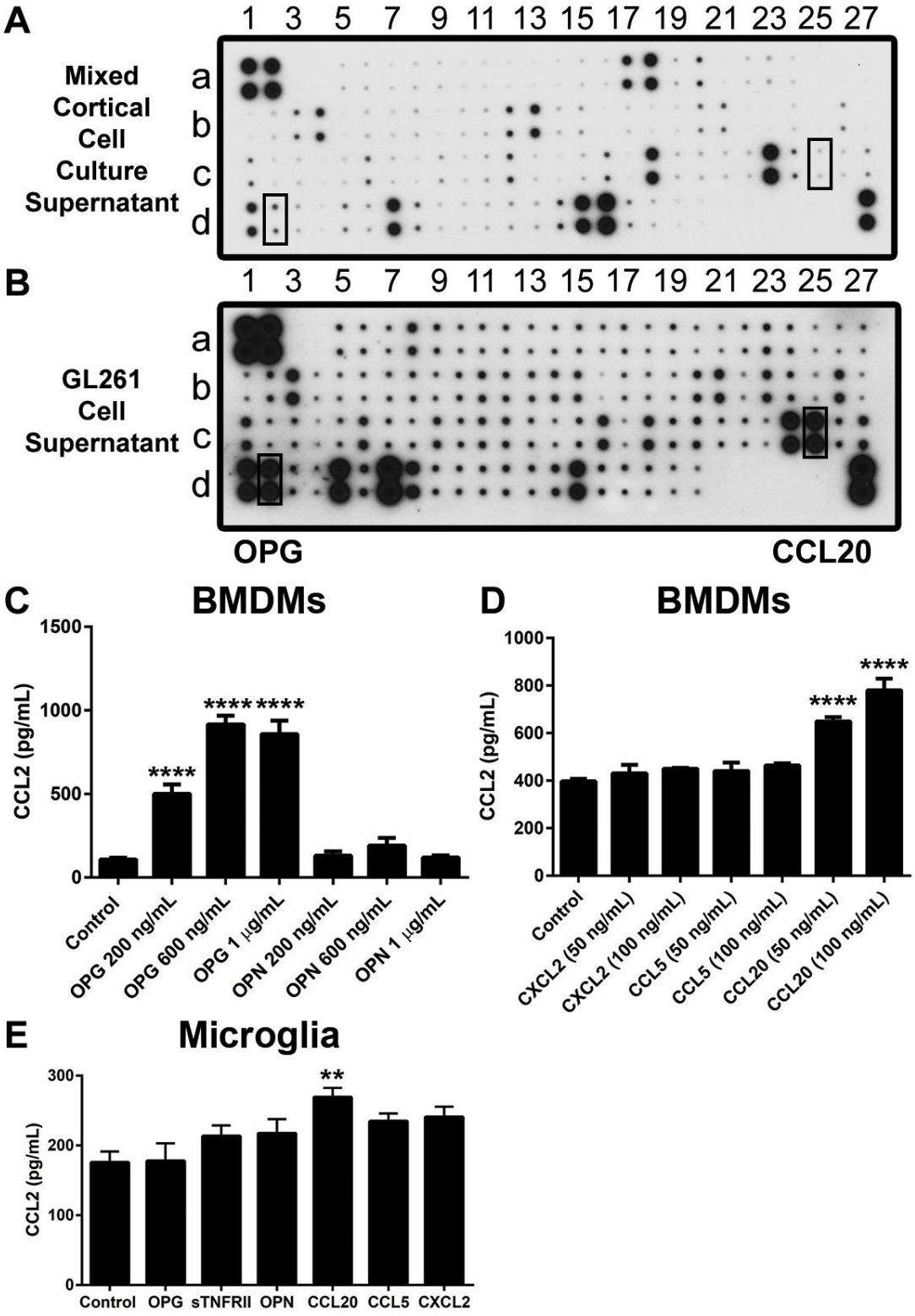

Figure 12. Glioma-derived osteoprotegerin and CCL20 induce CCL2 production from macrophages.

Figure 12, continued.
(A-B) Mouse cytokine array of MCCC or GL261 supernatant. (C-D) CCL2 secretion from BMDMs treated with candidate cytokines/chemokines identified from the antibody arrays (n = 3-6). (E) CCL2 secretion from microglia treated with candidate cytokines/chemokines (n = 3). Data in C-E are representative of 3-4 independent experiments. Data are represented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ by ordinary one-way ANOVA with Tukey's multiple comparisons test.

Table 1. Antibody Array Key (RayBiotech Cat# AAM-CYT-6)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	POS	POS	NEG	NEG	6Ckine	ALK-1	AREG	Axl	BLC	CT-1	CD27	CD27 Ligand	CD30	CD30 Ligand
	POS	POS	NEG	NEG	6Ckine	ALK-1	AREG	Axl	BLC	CT-1	CD27	CD27 Ligand	CD30	CD30 Ligand
B	Flt-3 Ligand	Fractalkine	Galectin 1	GAS 6	G-CSF	GITR	GITR Ligand	Granzyme B	HAI-1	HGF	IFN gamma	IGFBP 5	IGFBP 6	IGF-2
	Flt-3 Ligand	Fractalkine	Galectin 1	GAS 6	G-CSF	GITR	GITR Ligand	Granzyme B	HAI-1	HGF	IFN gamma	IGFBP 5	IGFBP 6	IGF-2
C	IL-12 p70	IL-13	IL-15	IL-17	IL-17B R	IL-17E	IL-17F	IL-20	IL-21	I-TAC	JAM-A	KC	Leptin	Leptin R
	IL-12 p70	IL-13	IL-15	IL-17	IL-17B R	IL-17E	IL-17F	IL-20	IL-21	I-TAC	JAM-A	KC	Leptin	Leptin R
D	OPN	OPG	PRL	Pro MMP-9	RANTES	SCF	sTNFRI	sTNFRII	TACI	TARC	TNF alpha	TPO	TRANCE	TROY
	OPN	OPG	PRL	Pro MMP-9	RANTES	SCF	sTNFRI	sTNFRII	TACI	TARC	TNF alpha	TPO	TRANCE	TROY

	15	16	17	18	19	20	21	22	23	24	25	26	27
A	CD36	CTLA-4	CXCL16	Decorin	Dkk-1	E Cadherin	EGF	Eoxtaxin 1	Eotaxin 2	Epigen	E Selectin	Fas Ligand	FcgRIIB
	CD36	CTLA-4	CXCL16	Decorin	Dkk-1	E Cadherin	EGF	Eoxtaxin 1	Eotaxin 2	Epigen	E Selectin	Fas Ligand	FcgRIIB
B	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-2R alpha	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-11	IL-12 p40
	IL-1 alpha	IL-1 beta	IL-1ra	IL-2	IL-2R alpha	IL-3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-11	IL-12 p40
C	L Selectin	Lungkine	Mad CAM-1	MCP-1	MDC	MFG E8	MIG	MIP-1 alpha	MIP-1 gamma	MIP-2	MIP-3 alpha (CCL20)	MIP-3 beta	MMP-2
	L Selectin	Lungkine	Mad CAM-1	MCP-1	MDC	MFG E8	MIG	MIP-1 alpha	MIP-1 gamma	MIP-2	MIP-3 alpha (CCL20)	MIP-3 beta	MMP-2
D	TWEAK R	VCAM-1	VEGF	VEGF R1	VEGF R	VEGF D	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS
	TWEAK R	VCAM-1	VEGF	VEGF R1	VEGF R	VEGF D	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS

Ccl2$^{-/-}$ ~1.26 x 10^4, p = 0.0072, mean absolute numbers of CD45^{high}CD11c$^-$CD11b$^+$Ly-6C$^+$ monocytic MDSCs) infiltrated the brain tumor in terms of absolute numbers (Figure 16A-16B). Interestingly, the number of infiltrating granulocytic Ly-6G$^+$ MDSCs remained unchanged despite the lack of CCL2 (Figure 16C). These results indicate that both Tregs and monocytic MDSCs (but not granulocytic MDSCs) require CCL2 for maximal recruitment to gliomas. In addition, these data further support our finding that the glioma microenvironment is the primary source of CCL2, since the GL261 cells implanted into *Ccl2*$^{-/-}$ mice should still be able to produce CCL2.

Tregs are disproportionately dependent on CCR4 for trafficking to glioma in comparison to effector T cells. Given CCR4 expression on Tregs and CD4$^+$Foxp3$^-$ T cells, we next determined the requirement for CCR4 for brain tumor trafficking. We generated mixed-bone marrow chimeras using bone marrow from wild-type CD45.1$^+$ and CD45.2$^+$ *Ccr4*$^{-/-}$ mice, injected the chimeras with GL261 cells, and analyzed the tissues after 1 week (Figure 17A). A slight deficiency was observed across all T cell populations within CD45.2$^+$ cells in the brain: Treg (CD45.1$^+$ WT 70.32 vs. CD45.2$^+$ *Ccr4*$^{-/-}$ 20.23, p < 0.0001, mean frequency), CD4$^+$Foxp3$^-$ (CD45.1$^+$ WT 57.34 vs. CD45.2$^+$ *Ccr4*$^{-/-}$ 31.17, p < 0.001), and CD8$^+$ (CD45.1$^+$ WT 56.45 vs. CD45.2$^+$ *Ccr4*$^{-/-}$ 33.20, p < 0.0001) (Figure 17B). However, Treg accumulation in the brain was disproportionately affected by the absence of CCR4, as both CD4$^+$Foxp3$^-$ T cell/Treg ratios were higher in CD45.1$^+$ cells compared to CCR4-deficient CD45.2$^+$ cells (CD45.1$^+$ WT 0.79 vs. CD45.2$^+$ *Ccr4*$^{-/-}$ 1.53, p = 0.0025, absolute number ratio) and CD8$^+$ T cell/Treg ratios (CD45.1$^+$ WT 0.61 vs. CD45.2$^+$ *Ccr4*$^{-/-}$ 1.29, p = 0.0018, absolute number ratio) (Figure 17C). CCR4 deficiency had the greatest impact on Treg accumulation in the brain, as the ratio of CD45.1$^+$/CD45.2$^+$ cells was significantly higher among brain tumor-infiltrating Tregs compared

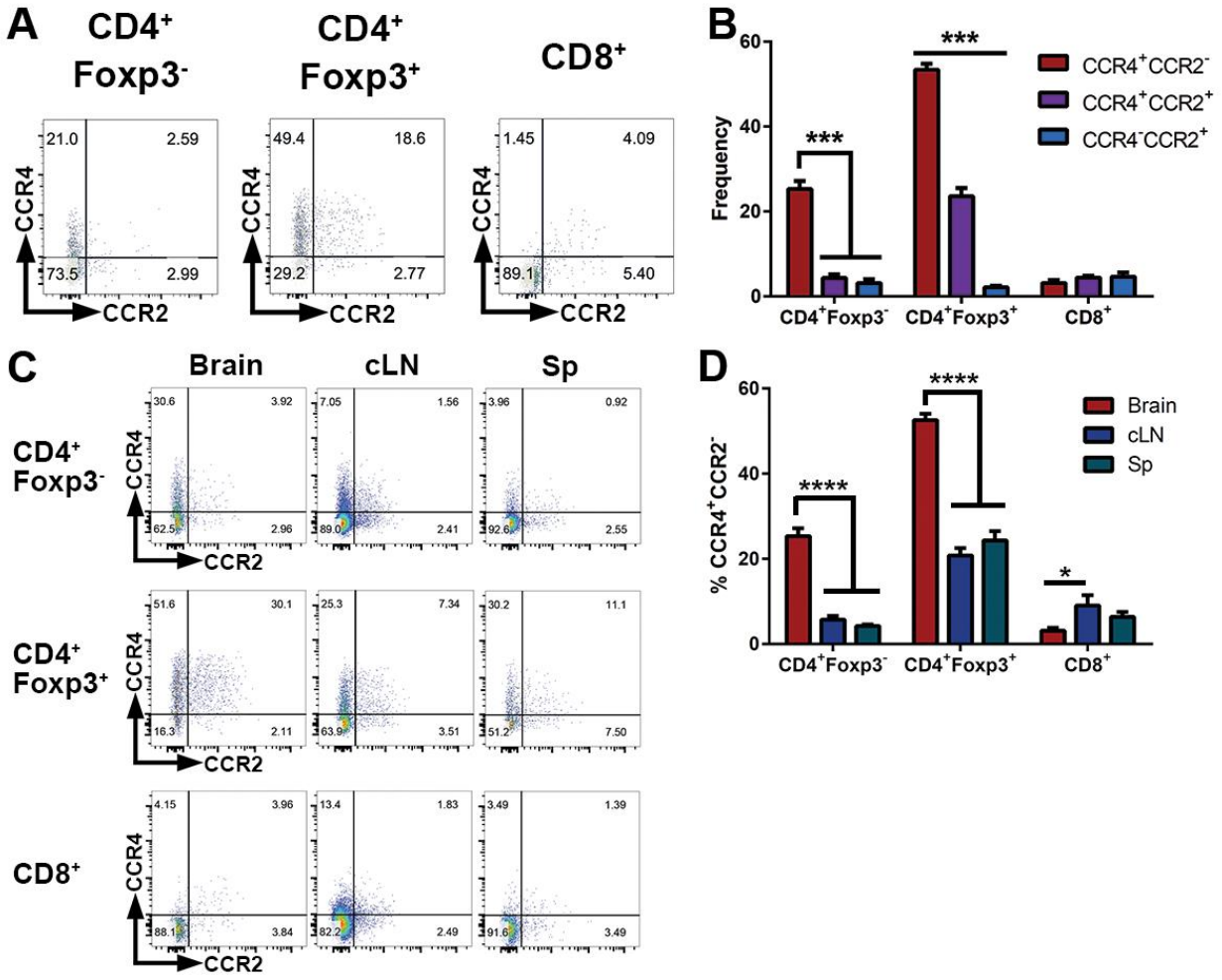

Figure 13. GL261 tumors are infiltrated by CCR4+ Tregs.
(A-B) Flow cytometric quantification of CCR4 and CCR2 expression among $CD4^+Foxp3^-$ T cells, $CD4^+Foxp3^+$ Tregs, and $CD8^+$ T cells (n = 5). (C-D) Quantification of CCR4 and CCR2 expression on T cell subsets in brain compared to cervical lymph nodes and spleen. Data are representative of at least 4 independent experiments. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by ordinary one-way ANOVA with Tukey's multiple comparisons test in B and D. cLN, cervical lymph nodes. Sp, spleen.

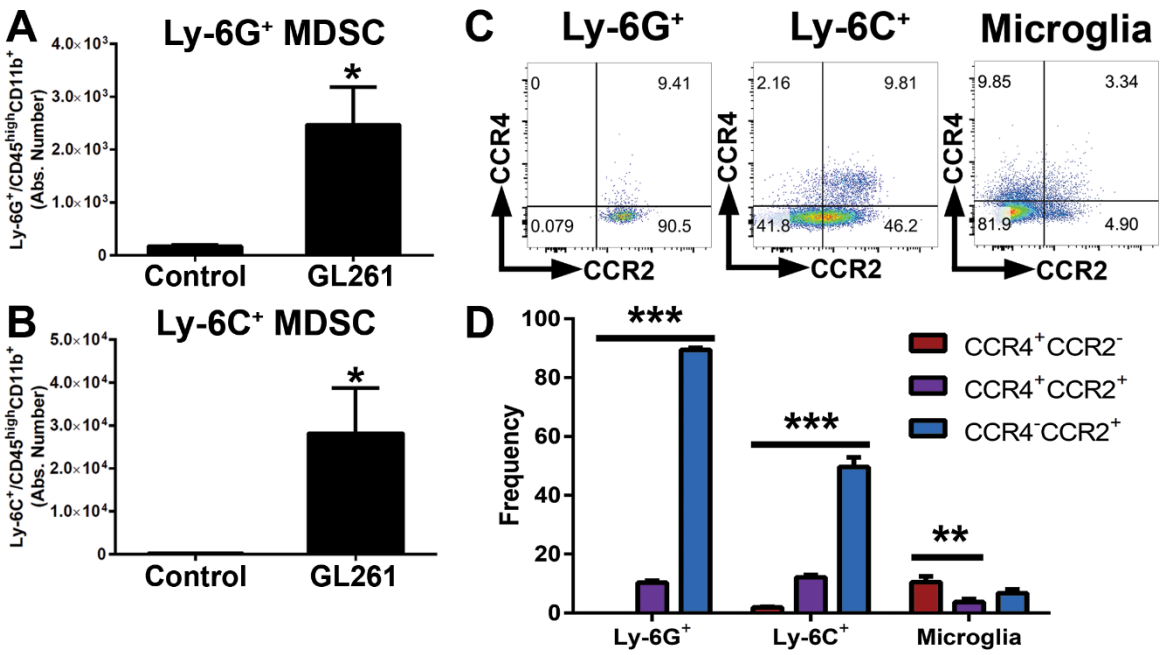

Figure 14. GL261 tumors are infiltrated by CCR2+ MDSCs.
(A-B) Absolute numbers of $CD11b^{+}Ly\text{-}6G^{+}$ and $CD11b^{+}Ly\text{-}6C^{+}$ cells in the brains of tumor bearing or PBS-injected mice (n = 5). (C) CCR4 and CCR2 expression in MDSC subsets and microglia. (D) Quantification of frequencies in C (n = 5). Data are representative of at least 4 independent experiments. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by ordinary one-way ANOVA with Tukey's multiple comparisons test in D or Student's *t* test in A-C.

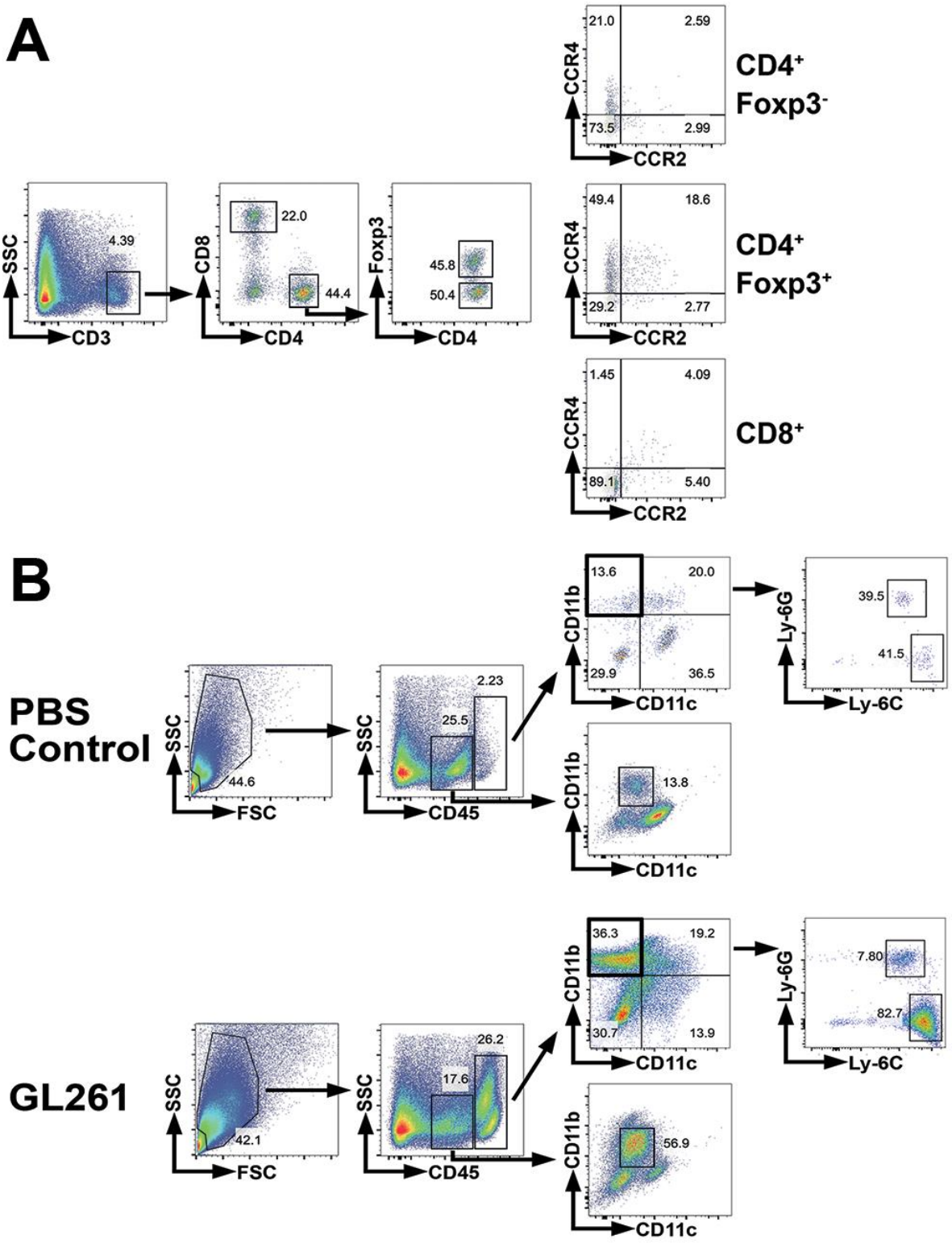

Figure 15. Gating strategy for flow cytometric analysis of T cells and myeloid cells in murine brain tumors.

Figure 15, continued.
(A) CCR4 and CCR2 expression on $CD4^+Foxp3^-$ conventional CD4 T cells, $CD4^+Foxp3^+$ Tregs, and $CD8^+$ T cells across various tissues at 1 week post-GL261 implantation. (B) Gating strategy for quantification of myeloid cells in brains of PBS-injected or GL261-implanted mice at 1-week post-intracranial implantation. Data are representative of at least 4 independent experiments.

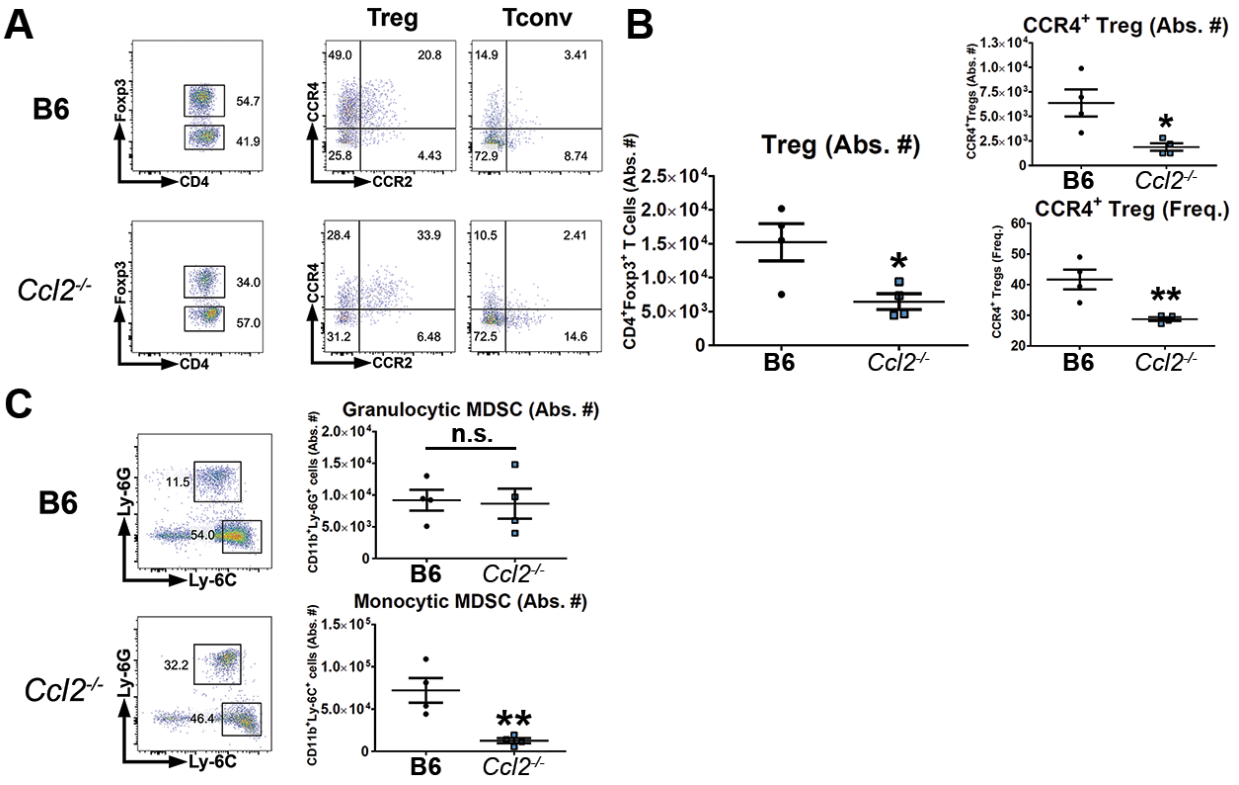

Figure 16. Regulatory T cell and myeloid-derived suppressor cell accumulation in CCL2-deficient mice.

(A-B) Treg infiltration at 1 week post-i.c. GL261 in wild-type C57BL/6 (B6) or *Ccl2*$^{-/-}$ mice. (*n* = 4). (C) MDSC infiltration in brain tumors of wild-type B6 mice or *Ccl2*$^{-/-}$ mice. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by Student's *t* test in B and C.

to mesenteric lymph nodes (mLNs), cLNs, and spleen (Figure 17D, Left). In contrast, the $CD45.1^+/CD45.2^+$ ratio among $CD8^+$ T cells was similar across tissues (Figure 17D, Right). Monocytic Ly-$6C^+$ MDSCs were not affected by CCR4 deficiency and granulocytic Ly-$6G^+$ MDSC numbers were slightly reduced in the absence of CCR4 (Figure 18). Taken together, these results suggest that CCR4 plays a role in the trafficking of T cells to the brain, but is selectively relevant to the recruitment of brain tumor-infiltrating Tregs.

The CCL2-CCR4 chemokine-chemokine receptor interaction is a potential therapeutic target in glioma. We hypothesized that targeting the CCL2-CCR4 interaction would decrease Treg recruitment in brain tumors. We first assessed the *in vitro* specificity and activity of the small molecule CCR4 antagonist C 021. C 021 treatment abrogated Treg chemotaxis to the CCR4 cognate chemokines CCL17 and CCL22 but did not affect Treg migration to RPMI containing 10% FBS and CCL21 (Figure 19A-19B). To determine the *in vivo* activity of C 021, we treated GL261-implanted mice with 15 mg/kg C 021 administered by subcutaneous injection every other day for five total doses. C 021-treated mice gained a 30% improvement in median survival over vehicle control-treated mice with a commensurate decrease of $CCR4^+$ Tregs and Ly-$6G^+$ MDSCs, whereas infiltrating $CD4^+$ and $CD8^+$ T cells were increased (Figure 20A-20G and 20J). In addition, both CD4:Treg and CD8:Treg ratios were improved in C 021-treated mice (Figure 20H-20I). Dose escalation experiments revealed that 15 mg/kg C 021 was sufficient to improve overall survival (Figure 20J). Thus, the CCR4 chemokine inhibitor selectively inhibits Treg accumulation with the benefit of improved median survival.

Monocytic Ly-6C+ MDSCs that lack CCR2 do not accumulate in glioma. Since CCR2 expression was largely localized to MDSCs, we performed a similar mixed-bone marrow chimera competition assay using wild-type $CD45.1^+$ bone marrow and $CD45.2^+$ CCR2-deficient

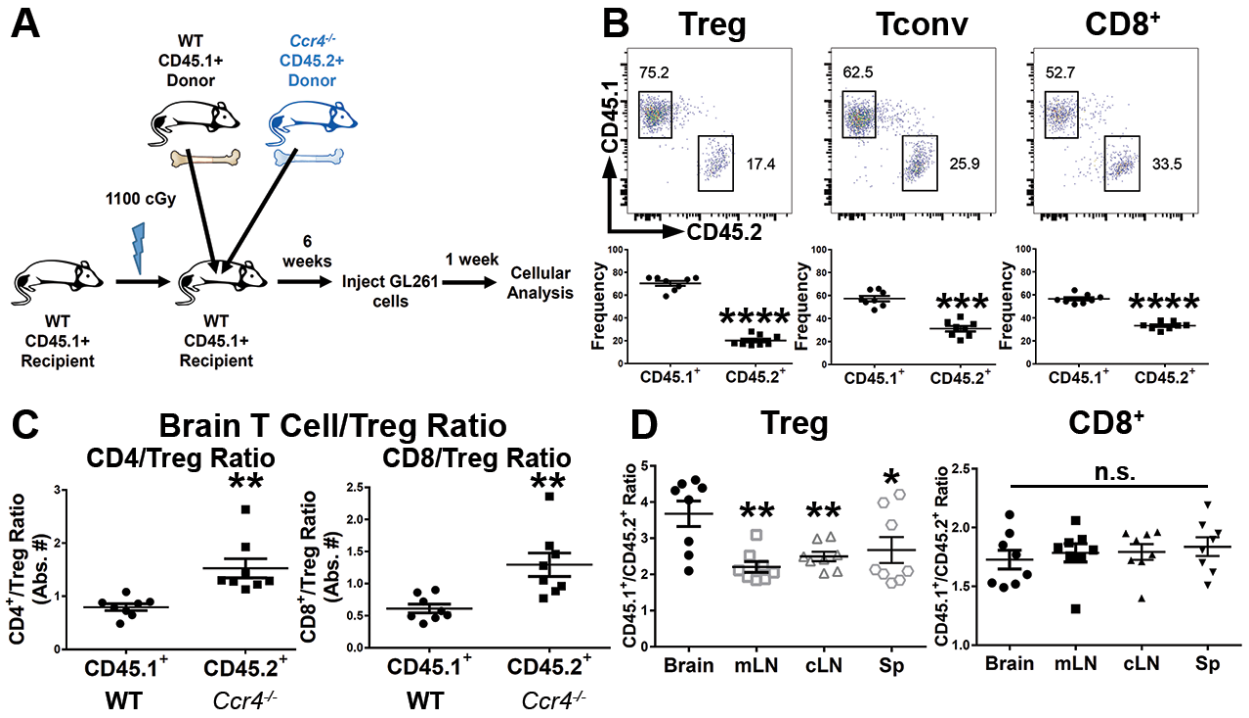

Figure 17. Influence of CCR4 chemokine receptor deficiency on T cell trafficking to gliomas.

(A) Schematic of mixed-bone marrow chimera (BMC) experiments using wild-type CD45.1$^+$ and CD45.2$^+$ *Ccr4*$^{-/-}$ marrow. (B) Flow cytometric analysis of BMC experiments of WT vs. *Ccr4*$^{-/-}$ cells within the T cell compartment. (n = 8) (C) CD4/Treg ratio and CD8/Treg ratio within CCR4-deficient cells or wild-type cells in brain tumor-bearing chimeric mice (n = 8). (D) CD45.1$^+$/CD45.2$^+$ cell ratios in Treg or CD8$^+$ T cell compartments across tissues in brain tumor-bearing chimeric mice (n = 8). Data are representative of 2-3 independent experiments. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by Student's *t* test in B and C or ordinary one-way ANOVA with Tukey's multiple comparisons test in D.

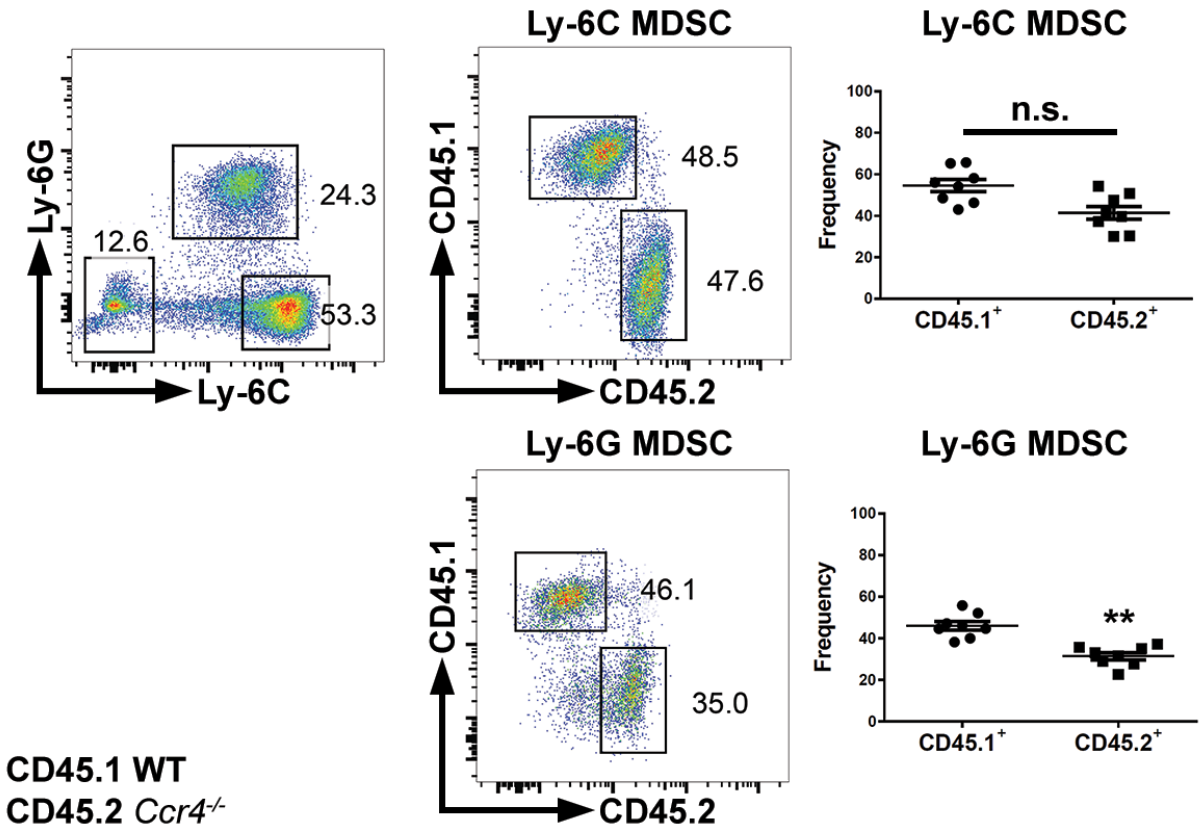

Figure 18. Myeloid-derived suppressor cell accumulation is partially affected by CCR4 deficiency.

$CD45.1^+$ recipient mice were irradiated and reconstituted with $CD45.1^+$ WT marrow and $CD45.2^+$ *Ccr4*$^{-/-}$ marrow. After at least 6 weeks of reconstitution, mice were implanted with GL261 tumors and brain tumor-infiltrating cells were quantified after 1 week. Data are representative of at least 2 independent experiments. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by Student's *t* test. n.s., not significant.

bone marrow (*Ccr2*$^{RFP/RFP}$) (Figure 21A) (Saederup et al., 2010). Within the CD45.2^{+} CCR2-deficient compartment, we observed an almost complete lack of Ly-6C^{+} monocytic MDSCs (Figure 21B). Ly-6G^{+} granulocytic MDSCs were unaffected by the absence of CCR2 in terms of trafficking to the brain tumor. Therefore, monocytic Ly-6C^{+} MDSCs require CCR2 in order for their ultimate accumulation in the GL261 tumors.

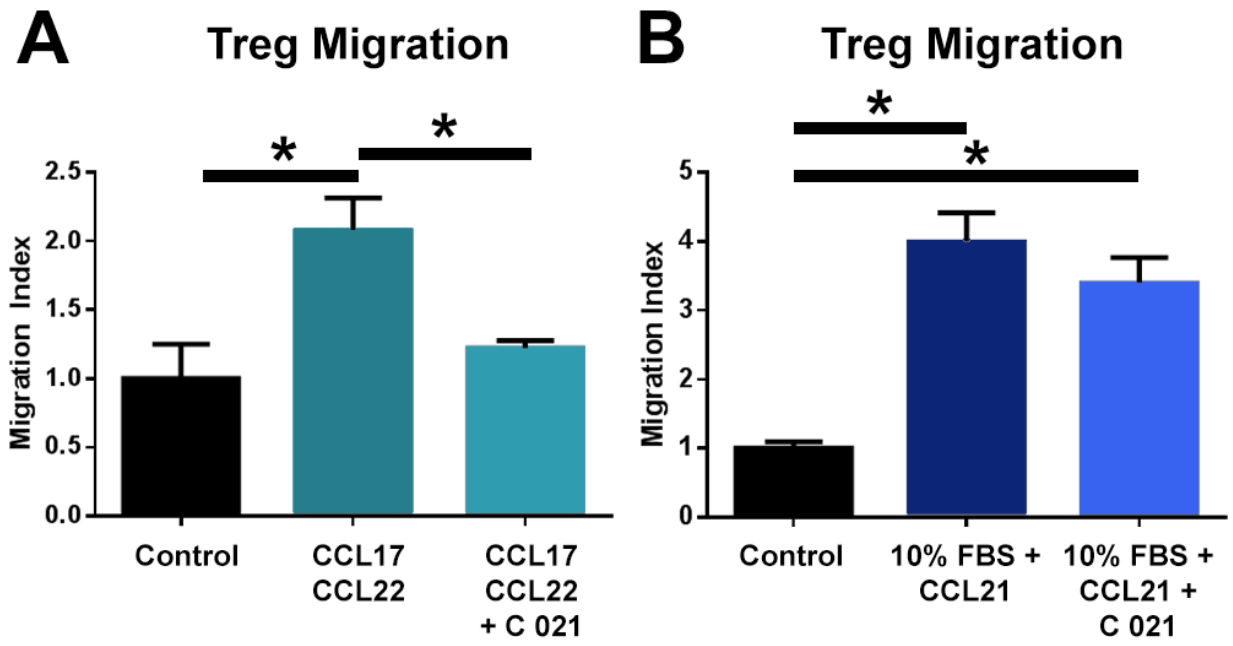

Figure 19. *In vitro* Treg migration in the context of the CCR4 antagonist C 021.
(A) Sorted Tregs were placed the upper chamber of a 3.0 µm pore cell culture insert with or without 5 µM of the small molecule antagonist C 021, and then chemokine-containing media was placed in the well. Migration index was determined after 4 hours through flow cytometric analysis. (B) A similar migration assay was performed as in A, except CCL21 and 10% FBS were used in the bottom chamber as a non-specific positive control. Data are representative of 3 independent experiments. Data are represented as mean ± SEM; *$p < 0.05$, by Student's *t* test.

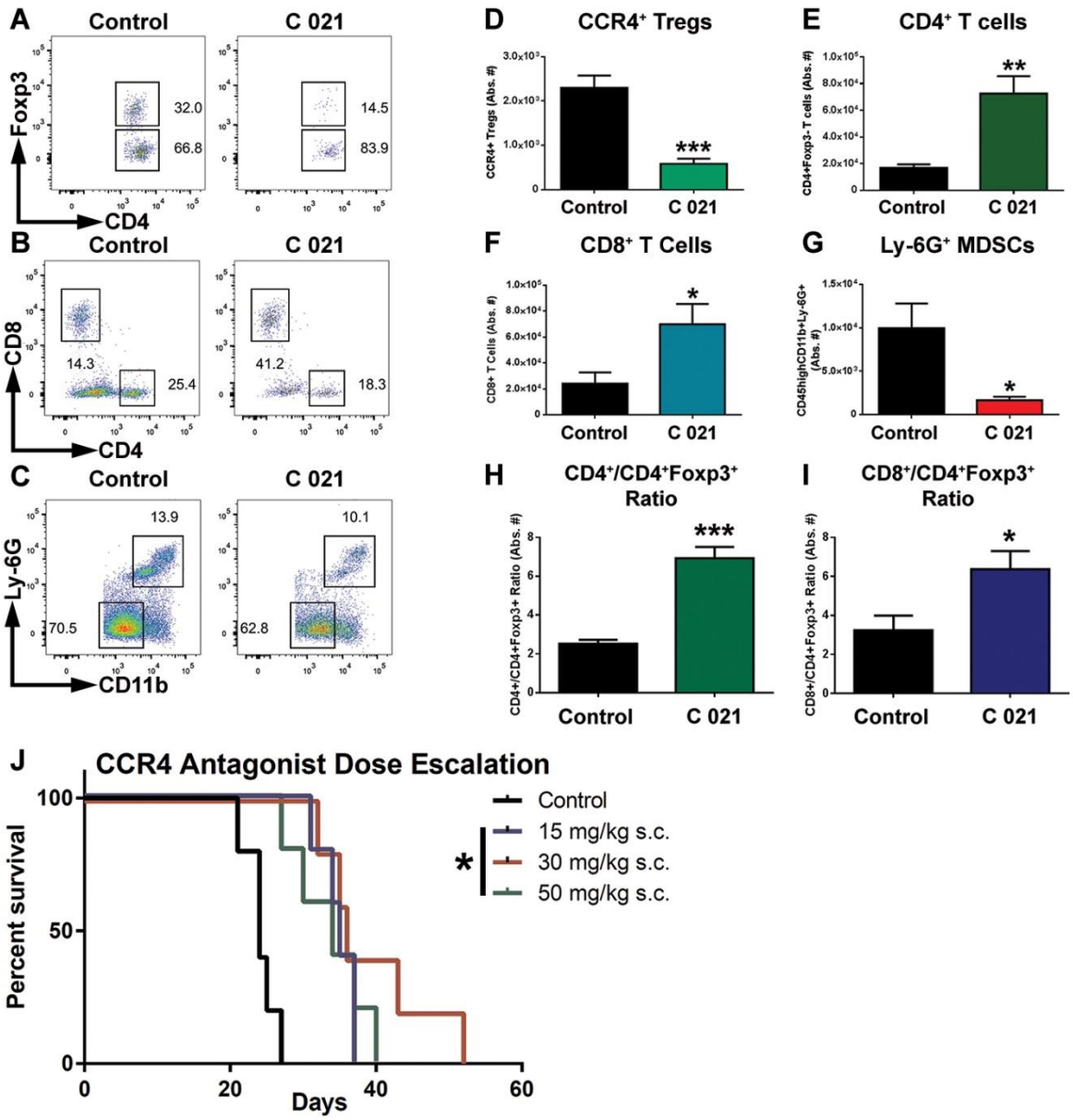

Figure 20. The small molecule CCR4 inhibitor C 021 influences immune cell infiltration and improves animal survival in GL261 tumors.

(A-C) Vehicle control or C 021-treated mice were analyzed at 1 week post-GL261 injection for the accumulation of various T cell subsets and $CD11b^{+}Ly\text{-}6G^{+}$ MDSCs in the brain. (D-G) Quantification of flow cytometric analysis. (H-I) CD4/Foxp3 ratio and CD8/Foxp3 ratio of control or C 021-treated mice. Data are representative of at least 2 independent experiments. (J) End-point analysis of mice implanted with GL261 cells and treated on alternating days with indicated doses of CCR4 antagonist (C 021) for a total of 5 doses. Data are representative of 2-3 independent experiments. Data are represented as mean ± SEM; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ by Student's *t* test. Kaplan-Meier survival curves were compared with the Log-rank test with the Bonferroni method for multiple comparisons.

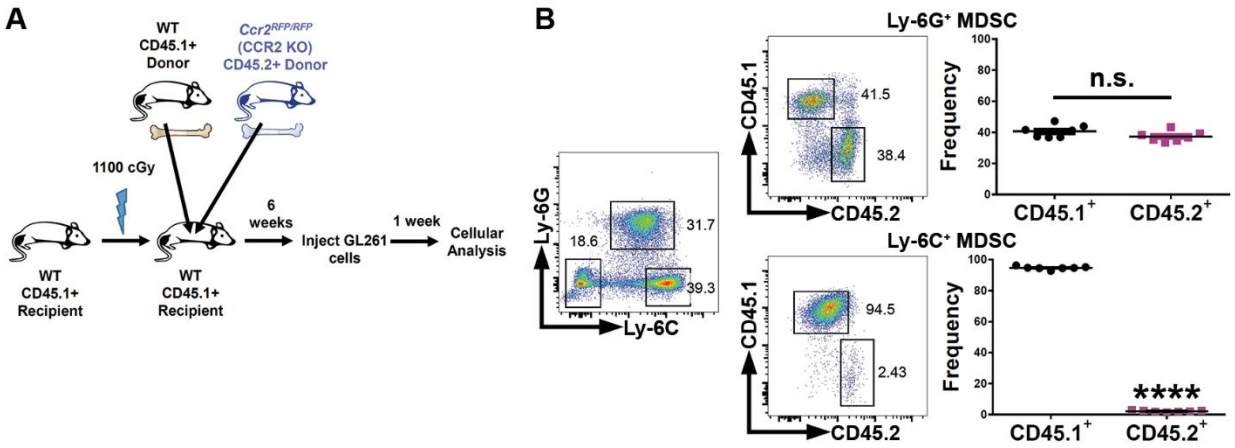

Figure 21. Ly-6C+ monocytic MDSCs require CCR2 for accumulation in glioma.
(A) Schematic of mixed-bone marrow chimera experiments using wild-type CD45.1$^+$ and CD45.2$^+$ *Ccr2*$^{RFP/RFP}$ (CCR2-deficient) marrow. (B) Wild-type CD45.1$^+$ or CCR2-deficient CD45.2$^+$ glioma-infiltrating MDSC subsets in brain tumor-bearing chimeric mice (n = 7). Data are representative of at least 2 independent experiments. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 by Student's *t* test.

Chapter IV. Discussion

This chapter has been adapted and expanded from the discussion section of Chang A.L., Miska J., Wainwright D.A., Dey M., Rivetta C.V., Yu D., Kanojia D., Pituch K.C., Qiao J., Pytel P., Han Y., Wu M., Zhang L., Horbinski C.M., Ahmed A.U., and Lesniak M.S. 2016. CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. *Cancer Research*. 76(19):5671-5682.

Overview

The immunosuppressive tumor microenvironment remains a major obstacle that impedes productive anti-tumor immune responses. Here, we have dissected a novel mechanism for Treg and MDSC recruitment that begins with tumor-derived CCL20 and osteoprotegerin which induces CCL2 production from glioma-associated macrophages and microglia (Figure 22). This mechanistic axis illustrates the potential of macrophages as an additional source of CCL2 in GBM patients. In the tumor microenvironment, astrocytoma cells are not only capable of producing CCL2 themselves but are also able to induce glioma-associated myeloid cells to secrete CCL2. Importantly, tumor-derived CCL2 and macrophage-derived CCL2 are not necessarily mutually exclusive possibilities. CCL2 then recruits Tregs and MDSCs through CCR4 and CCR2, respectively, as major contributors to the potently immunosuppressive glioma microenvironment.

DISCUSSION

Pro-tumorigenic versus anti-tumorigenic roles of CCL2.

The pro-tumorigenic or anti-tumorigenic effects of CCL2 are heavily context-dependent. CCL2-mediated recruitment of MDSCs, monocytes, and macrophages supports colorectal

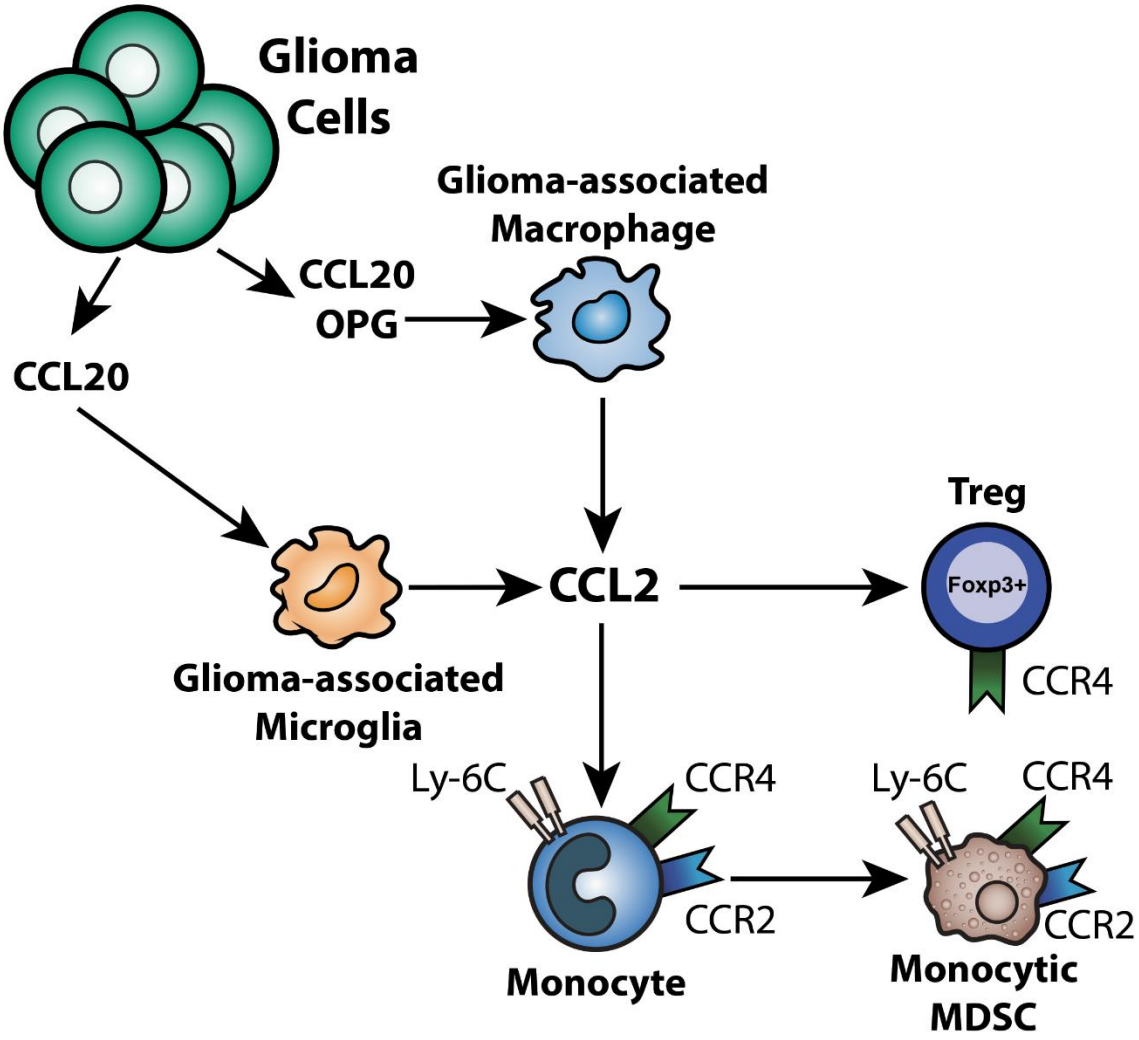

Figure 22. A model describing the CCL2-CCR4/2 axis in GBM.
Glioma-derived CCL20 and OPG induces the production of CCL2 from glioma-associated macrophages and microglia. CCL2 recruits CCR4-expressing Tregs and CCR2-expressing monocytes to GBM. These monocytes then differentiate into $CCR2^{+}$ MDSCs.

carcinogenesis and endometrial cancer growth (Chun et al., 2015; Pena et al., 2015). In breast cancer and lung cancer metastases, CCL2-CCR2 interactions recruit metastasis-associated macrophages as well as monocytes which in turn promote metastasis in an E-selectin-dependent manner (Hauselmann et al., 2016; Kitamura et al., 2015; Qian et al., 2011). In addition, CCR2-expressing Tregs enhance tumorigenesis in mouse models using MCA-induced sarcomas, MMTV-PyMT murine mammary tumors, and in oral squamous cell carcinoma patients (Loyher et al., 2016). In contrast, CCL2 can act to recruit cells with anti-tumor capacity. CCL2-recruited monocytes degrade fibrosis in an IFNγ-dependent manner in the context of anti-CD40 agonist treatment, improving gemcitabine efficacy in murine models of pancreatic carcinoma (Long et al., 2016). CCL2 can also recruit functional antigen-presenting cells in the context of chemotherapy, in which immunogenic cell death is likely to be of considerable significance (Ma et al., 2014). Thus, tumors with high amounts of CCL2 may be poised for the induction of a productive immune response if sufficient IFNγ or strongly immunogenic cell death can be induced. In the absence of such initiation, the monocytes and macrophages recruited by CCL2 instead contribute to the immunosuppressive microenvironment together with Tregs trafficking in response to CCL2.

A potential function for CCR4 in early T cell development.

In our mixed-bone marrow chimera studies, we observed some reduction in numbers across Treg, $CD4^+Foxp3^-$, and $CD8^+$ T cell populations within $CD45.2^+$ *Ccr4*$^{-/-}$ cells compared to $CD45.1^+$ wild-type cells. Interestingly, this may reflect a minor role for CCR4 in an earlier developmental stage. Aire-dependent expression of CCR4 occurs during the development of multiple αβ T cell lineages in the thymus, although CCR4 is not unanimously found to be required for T cell development (Campbell et al., 1999; Cowan et al., 2014; Laan et al., 2009). In

addition, CCR4 enhances thymocyte medullary entry (Bleul and Boehm, 2000). In this study, CCL22 and CCL17 were expressed by myeloid cells in the medulla and CCR4 mediated thymocyte chemotaxis *in vitro*. Later mechanistic work demonstrated a potential role for CCR4 in influencing tolerance mechanisms and CCR4 ligand expression in thymic SIRPα DCs (Hu et al., 2015). The role of CCR4 in thymocyte migration and tolerance mechanisms may be recapitulated post-thymic development as CCR4 mediates trafficking of specific T cell subsets to sites of inflammation and/or tumors.

TCR specificity of glioma-infiltrating Tregs.

The presence of Treg infiltration in certain GBM patients raises an interesting question of the TCR specificity of glioma-infiltrating Tregs. Physical barriers to cell entry and limited antigen drainage appear to be major mechanisms of attenuating immune reactivity in the CNS under normal physiological conditions. In addition, Tregs have long been implicated as critical mediators of negative immune regulation during CNS inflammation, such as in experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis (Kleinewietfeld and Hafler, 2014). In EAE, transfer of myelin basic protein (MBP)-specific Tregs is protective against the induction of disease (Stephens et al., 2009).

Several intriguing possibilities exist for the GBM Treg TCR repertoire. First, Tregs that are present in GBM may have TCR specificities for CNS antigens that have been unmasked to the immune system during tumorigenesis. Second, the Treg TCR repertoire in GBM may be specific to GBM tumor-associated antigens or tumor-specific antigens. The tumor-specific antigen possibility is less likely in the context of thymic Tregs since there is no point in time that the thymic Treg precursor would have encountered tumor-specific antigens during development and differentiation into the Foxp3-expressing Treg lineage. This, however, is possible in the context

of TCR-cross reactivity which could result in thymic Tregs with TCR specificity towards GBM tumor neo-antigens. Alternatively, the abundance of TGFβ in the glioma microenvironment may result in pTregs with TCRs recognizing tumor neo-antigens (Crane et al., 2012; Crane et al., 2010). The third possibility is that GBM-associated Tregs are brought into the CNS through mechanisms that operate somewhat independently of TCR specificity, including chemokines or the inherent "leakiness" of the BBB due to GBM neovascularization and tumor progression (Dhermain et al., 2010; Hardee and Zagzag, 2012; van Tellingen et al., 2015; Watkins et al., 2014). Elucidating the TCR specificity of glioma-associated Tregs may reveal more about why Treg infiltration is observed in some GBM patients and not others.

MDSC accumulation and CCR2 deficiency.

Our observation that CCR2-deficient MDSC accumulation did not occur in gliomas parallels other findings that CCR2 deficiency impairs circulating monocyte-dendritic cell progenitors and monocyte progenitors (Yona et al., 2013). Our analysis confirms a role for CCR2 in the accumulation of glioma-infiltrating monocytic Ly-6C^{+} MDSCs but does not absolutely decouple the requirement of CCR2 for monocyte trafficking/MDSC trafficking to glioma from that of an earlier monocyte progenitor role. Furthermore, differential MDSC subset sensitivity to both CCL2 and CCR2-deficiency is meaningful given that the monocytic MDSC lineage was recently identified as more immunosuppressive in EG7 lymphoma, Lewis lung carcinoma, and B16 melanoma tumors (Haverkamp et al., 2014). Further characterization of MDSCs in GBM patients remains an area of considerable interest.

CCR4 and CCR2 as a clinical targets for therapy.

The therapeutic benefit of CCR4 inhibition highlights the potential for other modalities of targeting CCR4, such as therapeutic antibodies. Mogamulizumab, a defucosylated humanized

anti-CCR4 antibody with demonstrated efficacy against T cell lymphomas, has been adapted for targeting Tregs in solid tumors in a Phase Ia clinical trial (Duvic et al., 2015; Kurose et al., 2015; Ogura et al., 2014). Treatment with mogamulizumab in patients with adult T cell leukemia-lymphoma and peripheral T cell lymphomas results in depletes $CCR4^+$ cells for several months, a testament to the potency of this antibody therapy (Yamamoto et al., 2010). In a cohort of lung and esophageal cancer patients, mogamulizumab preferentially depleted Tregs even at the lowest dose, with some depletion effects on Th2 and Th17 CD4 T cells, which can also express CCR4 (Kurose et al., 2015). Alternatively, CCR4 can be targeted through an immunotoxin approach – this concept was recently validated in a non-human primate model using CCR4 conjugated to diphtheria toxin (Wang et al., 2016).

Small molecule inhibition of CCR2 as a therapeutic strategy to target tumor-associated macrophages was recently evaluated in a Phase Ib clinical trial in pancreatic ductal adenocarcinomas (Nywening et al., 2016). In this study, the small molecule CCR2 inhibitor PF-04136309 was found to be safe when used in combination with the FOLFIRINOX chemotherapy regimen (oxaliplatin and irinotecan plus leucovorin and fluorouracil). CCR4/CCR2 targeting is likely to be most effective in combination with immunotherapeutic approaches that activate the effector response, such as checkpoint blockade or vaccination in order to overcome immunosuppression at the tumor site while preventing the additional recruitment of immunosuppressive cells.

The presence of immune infiltration in GBM.

CCL2 is likely part of a larger gene expression signature that determines the presence of TIL/MDSC/Treg infiltration in GBM (Han et al., 2014; Rutledge et al., 2013; Sayour et al., 2015; Thomas et al., 2015; Yue et al., 2014). Identifying factors underlying Treg, MDSC, and

TIL recruitment in brain tumors is vital for the strategic disarming of critical immune evasion pathways. Although there is evidence that the mesenchymal molecular subtype of glioblastoma is characterized by the presence of TILs, the immunophenotype of glioblastomas remains understudied (Rutledge et al., 2013). The correlation of immune cell infiltrate with GBM transcriptional subclass is complicated by the existence of multiple transcriptional subtypes within a single tumor, along with longitudinal changes in molecular subtype with tumor progression and recurrence (Kim et al., 2015; Sottoriva et al., 2013). It remains to be seen how immune phenotype changes longitudinally as well as how closely immune infiltrate is linked to transcriptional subtype. Furthermore, it may be important to expand the coverage of immune cell phenotyping in order to identify key immune cells that influence anti-tumor immunity in GBM. This objective could be accomplished with current mass cytometry methods which enable fairly extensive identification of immune cell subsets, cellular phenotype, and immune cell activation status (Spitzer and Nolan, 2016). Alternatively, computational methods exist which are capable of inferring the presence and proportion of immune cell subsets from gene expression data. The CIBERSORT and PRECOG methods have been used to infer the presence of immune cell infiltration based on gene expression and the prognostic value of various immune signatures (Gentles et al., 2015; Newman et al., 2015). How the presence of immune cells influence survival in GBM patients, which immune cell subsets impart the greatest impact on patient survival, and the determinants of immune cell infiltration remain critical open questions to be addressed as immunotherapies for GBM are developed in clinical trials.

FUTURE DIRECTIONS

CCL2-mediated cell recruitment in the context of radiation and immunotherapeutic approaches.

We elucidated how CCL2 recruits Tregs and monocytic MDSCs in the GL261 model, further identifying how chemokine receptor inhibition could work as a therapeutic intervention. An area of future interest would be how CCL2 production is influenced in the context of other therapeutics. Given that CCL2 can function as a pro-inflammatory cytokine, radiotherapy may be able to influence CCL2 or shift the balance in favor of productive immune responses. Additionally, the CCL2 chemokine-receptor axis could be examined in the context of immunotherapy such as checkpoint blockade. This could be studied through the comparing the GL261 cell line, which is sensitive to checkpoint blockade, with the CT2A cell line, a glioma cell line syngeneic to the B6 background that is relatively refractory to checkpoint blockade (Reardon et al., 2016; Wainwright et al., 2014). CCL2 may also play a key role in the context of standard therapeutic interventions for GBM. A Luminex cytokine screen of GBM patient plasma pre- and post- surgery following intraoperative high-dose tumor bed radiotherapy found high levels of CCL2 in a subset of patients (Frank A. Giordano, personal communication). High serum levels of CCL2 could be an important clinical biomarker for those patients who may benefit from immunotherapeutic intervention following radiotherapy as an indication of productive anti-tumor responses.

Inducing productive inflammatory immune responses in gliomas: the role of radiation or other chemotherapeutic agents in immunogenic cell death.

The extent to which glioblastoma cells are recognized by the immune system is incompletely understood. The total level of mutation burden in GBM is relatively low in comparison with other cancer types – indicating that the number of potential tumor neo-antigens would be reduced in GBM (Alexandrov et al., 2013; Lawrence et al., 2013). Thus, even when T cells can surmount the physical blockade of the blood brain barrier, potent tumor neo-antigens capable of immune

recognition may not exist. Another aspect of GBM therapy that lacks clarity is the extent to which current chemotherapy and radiotherapy approaches cause immunogenic cell death. A sufficiently high intratumoral concentration of TMZ elicits immunogenic cell death in a T cell dependent manner when combined with a model vaccine strategy (Fritzell et al., 2013). The extent to which other chemotherapeutic agents used in glioma treatment, such as 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), compare in terms of immunogenic cell death to TMZ is also worthy of investigation. In terms of radiation for glioma therapy, immunogenic cell death could be studied using more precise small animal irradiation apparatuses to better model radiotherapy treatment plans used in the clinic. The subanatomical locations of GBM may influence immunogenic cell death. Despite the fact that the GBM tumor core thought to be extensively hypoxic and necrotic, it is unknown whether this characteristic indicates that immunogenic tumor cell death is occurring in these regions.

Clinical biomarkers of immune response in GBM.

The importance of CCL2 as a biomarker extends two other major questions in the field of glioma tumor immunology: determinants of immune cell infiltration in GBM patients and the balance between productive immune response versus deleterious inflammation/edema in GBM. With immunotherapy for melanoma, it has become evident that certain biomarkers are crucial for identifying those patients most likely to respond to anti-CTLA4, and anti-PD-1/PD-L1 blockade. These markers include IFNγ pathway gene expression, $CD8^+$ T cell infiltration at infiltrative tumor borders, mutational load, and high levels of tumoral PD-L1 expression (Gao et al., 2016; Taube et al., 2012; Topalian et al., 2016; Tumeh et al., 2014). The mutational landscape has also been implicated as a major factor for sensitivity to PD-1 blockade in non-small cell lung cancer (Rizvi et al., 2015). In GBM, PI3K-Akt pathway activation as a result of PTEN mutations drive

PD-L1 expression (Parsa et al., 2007). Clinical trials designed to test the safety and efficacy of nivolumab (anti-PD-1) and ipilimumab (anti-CTLA4) in GBM found that the combination of both antibody therapeutics significantly increased the risk for toxicity (Curry and Lim, 2015). Given the link between inflammation, edema, and clinical degradation in GBM, it is of great interest which biomarkers may be able to predict clinical response to immunotherapy in GBM and whether any markers exist which differentiate between response, pseudoprogression, and edema risk.

How CCL2 and other cytokines are a part of IFNγ gene signature could be determined through Gene set enrichment analysis (GSEA) or pathway analysis of the GBM datasets could determine how CCL2 and other cytokines are part of an IFNγ gene signature indicative of a T cell-inflamed tumor. This signature in combination with mutational burden information in the TCGA database may be key to understanding which GBM patients have the potential for a productive anti-tumor immune response with therapeutic intervention. Recent preliminary reports indicate a link between mutation load and response to checkpoint blockade in DNA repair-deficient GBM (two cases due to germline biallelic mismatch repair deficiency and one case due to a germline *POLE* mutation) (Bouffet et al., 2016; Johanns et al., 2016). In general, the mutational load of GBM is lower compared to cancers with high somatic mutation frequencies such as melanoma, lung squamous cell carcinoma, and lung adenocarcinoma (Alexandrov et al., 2013; Lawrence et al., 2013). Yet the wide range of mutation frequencies within GBM patients could differentiate patients regarding response to immunotherapy. However, mutational load alone does not appear to be sufficient to explain the presence of T cell infiltration. Two recent studies have found that while the PD-1 axis was correlated with the presence of host immune response, neither mutational load nor the presence of immunogenic antigens based on non-synonymous mutation

frequency were correlated with a T cell-inflamed tumor microenvironment (Danilova et al., 2016; Spranger et al., 2016). An interesting finding from this work is that tumors without an inherent T cell infiltration still possess tumor-associated antigens and tumor neoantigens capable of recognition by T cells. An important implication of this is that tumors devoid of T cells still have the potential for the induction of successful anti-tumor immune responses, if T cell infiltration can be achieved. Multiple factors contribute to T cell infiltration in GBM, which may include PD-1/PD-L1 axis expression, IFNγ expression signature, mutational burden, the presence of specific DC subsets, and/or specific mutational lesions. The elucidation of these factors will be key for the understanding of how anti-tumor immunity can be achieved in GBM.

The CIBERSORT and PRECOG results provide some intriguing insight into which immune cell subsets are potentially critical for anti-tumor immunity in GBM. The training dataset used to develop the CIBERSORT algorithm did not include gene expression signatures for resting microglia or activated microglia. Whether microglial gene expression signatures can be clearly delineated from various macrophage gene expression signatures and whether these immune cell signatures may be important for GBM would require further refinement of the algorithms. Applying the CIBERSORT and PRECOG algorithmic methods to the TCGA GBM dataset results in three immune cell infiltration signatures with prognostic value: macrophages, memory CD4 T cells, and naïve B cells (Gentles et al., 2015). Of these signatures, the naïve B cell signature is associated with a favorable prognosis whereas memory CD4 T cell infiltration and macrophage infiltration are associated with worse prognosis. How these cellular subsets mechanistically contribute to anti-tumor immunity or hinder the immune response in GBM remain open questions for future investigation.

The influence of OPG and CCL20 on CCL2 production from macrophages.

We found that CCL2 production from macrophages could be mediated by tumor-derived OPG and CCL20. CCL20 enhances cytokine production from osteoblasts in a manner that is partially dependent on IL-6 (Pathak et al., 2015). Additionally, peritoneal macrophages in CCR6-deficient mice express lower levels of pro-inflammatory cytokines and nitric oxide following LPS stimulation (Wen et al., 2007). How CCL20 and IL-6 induce macrophage-derived CCL2 production in the glioma microenvironment remains an interesting question to address. OPG is a molecule which antagonizes RANK-RANKL signaling by sequestering free RANKL, which raises the possibility that the RANK axis additionally influences macrophage function by influencing chemokine production. In the context of ischemia, OPG/RANKL/RANK signaling is a key mediator of inflammation (Shimamura et al., 2014). In this work, the authors found that increased OPG was tied to increased inflammation in the ischemic brain through increasing pro-inflammatory cytokine secretion from macrophages and microglia. This signaling axis may play an important role in the glioma microenvironment by as part of the non-resolving inflammatory microenvironment that ultimately supports tumor growth.

CONCLUSION

Our findings highlight the impact of microenvironment-derived CCL2 in the accumulation of immunosuppressive cells and provide additional critical insight into the barriers that must be overcome for the successful translation of immunotherapies to treat GBM. With each advance in understanding of brain tumor immunity, major strides can be made toward the next era of treatment for brain tumors.

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