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CCL5, CCR1 and CCR5 in murine glioblastoma: immune cell infiltration and survival rates are not dependent on individual expression of either CCR1 or CCR5

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Abstract

Glioblastoma multiforme (GBM) is the most malignant brain tumor. Microglia/macrophages are found within human GBM where they likely promote tumor progression. We report that CCL5, CCR1, and CCR5 are expressed in glioblastoma. Individual deletion of CCR1 or CCR5 had little to no effect on survival of tumor bearing mice, or numbers of glioblastoma-infiltrated microglia/macrophages or lymphocytes. CCL5 promoted *in vitro* migration of wild type, CCR1- or CCR5-deficient microglia/macrophages that was blocked by the dual CCR1/CCR5 antagonist, Met-CCL5. These data suggest that CCL5 functions within the glioblastoma microenvironment through CCR1 and CCR5 in a redundant manner.

Keywords

glioblastoma; GL261; microglia; Met-CCL5; RANTES

1. Introduction

Among more than 120 types of brain tumor, glioblastoma multiforme (GBM) is the most aggressive and lethal form due to its rapid growth rate, high invasiveness to surrounding normal tissues, and ability to escape the host immune system (Kelly et al. 1984, Prados, 2000, Furnari et al., 1995, Kleihues et al., 1995, Holland, 2001). Although several therapeutic approaches have been applied, including surgery, radiotherapy, and chemotherapy, none of these treatments are very effective at halting tumor progression (Marks, 1989, Mangiola et al., 2010, Hosli et al., 1998, Nicholas et al., 2011). Of interest, a large proportion of microglia/macrophages cells are found within this type of malignant human glioma as well as mouse models of the disease (Graeber et al., 2002, Liu et al., 2009, Charles et al., 2011). Generally, these myeloid-derived, macrophage-like microglial cells function as the main form of immune defense in the central nervous system (Gehrmann et al., 1995, Streit, 2001, Alliot et al., 1999, Chan et al., 2007, Rivest, 2009). When activated by pathological changes in the brain, they transform from a resting state to a pro-inflammatory phenotype that is capable of phagocytosis, cytotoxicity, and antigen

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presentation through the high expression of major histocompatibility complex (MHC) class II molecules (Davalos et al., 2005, Fetler and Amigorena, 2005, Gehrmann et al., 1995, Nimmerjahn et al., 2005, Stoll et al., 2006). Opposite to their normal function in the immune system, glioma infiltrated microglia/macrophages show an anti-inflammatory phenotype, in which the expression of MHC class II molecules is down-regulated, especially in brain tumors with high malignancy (Badie et al., 2002, Bettinger et al., 2002, Flügel et al., 1999, Morantz et al., 1979, Tran et al., 1998, Badie and Schartner, 2001, Watters et al., 2005, Roggendorf et al., 1996), possibly by glioma-secreted factors, including IL-4, IL-6, IL-10, transforming growth factor β (TGF β), and prostaglandin E₂, (Charles et al., 2011). A positive correlation between the number of infiltrating microglia/macrophages and the proliferation rate of the tumor is also evident (Graeber et al., 2002, Morimura et al., 1990). Thus, these immune cells are believed to contribute to the local immunosuppressive milieu of glioma, as well as promote tumor progression (Hussain et al., 2006, Yang et al., 2010, Markovic et al., 2005). Despite the potential importance of microglia/macrophages in glioma tumorigenesis, the mechanism by which microglia/macrophages infiltrate into the tumor is still unknown. Increasing evidence supports the hypothesis that glioma regulates this mechanism through different factors, including hepatocyte growth factor/scatter factor (HGF/SF), colony-stimulating factor 1 (CSF-1), granulocyte-colony stimulatory factor (G-CSF) (Badie et al., 1999, Alterman and Standley, 1994, Nitta et al., 1992), or possibly MMP-2 and MMP-9, which are found abundantly within glioma (Nakada et al., 2003). Current studies strongly suggest this localization may require the presence of chemokines, a family of chemoattractant cytokines, in the tumor microenvironment (Sciumè et al., 2010, Kielian et al., 2002, Platten et al., 2003, Leung et al., 1997).

Due to their chemoattractant property, chemokines and chemokine receptors are well-known for guiding the migration of cells (Allen et al., 2007). During tumorigenesis, chemokine networks play important roles in many processes required for tumor development, such as tumor growth, proliferation, invasion, and angiogenesis (Balkwill, 2004, Kakinuma and Hwang, 2006, Koizumi et al., 2007, Mantovani et al., 2010, Bajetto et al., 2002). In the case of glioblastoma, treatment with the CXCR4 antagonist, AMD 3100, inhibits growth of murine intracranial glioblastoma and reduces the proliferation of tumor cells (Rubin et al., 2003). We have determined that the CXCR3 antagonist, NBI-74330, decreases tumor growth and prolongs survival in glioma-bearing mice (Liu et al., 2011). With regard to the interaction between microglia/macrophages and glioma, increasing evidence supports the participation of chemokines in glioma-microglia/macrophages crosstalk (Boehme et al., 2000, Semple et al., 2010). For example, CCL2 is a well described candidate for this function as an anti-CCL2 antibody prevented the migration of microglia/macrophages in response to CCL2 (Kielian et al., 2002, Platten et al., 2003). In contrast, recent evidence indicated that CCL7 (monocyte chemo-attractant protein-3), but not CCL2, is indeed the chemokine responsible for this function since CCL7 was predominantly expressed in glioma cell lines and the expression level of CCL7 is correlated to the percentage of GIM (glioma infiltrated microglia/macrophages) in glioma tissues (Okada et al., 2009).

Another member of the CC family, CCL5 (alternatively named RANTES) also promotes macrophage and lymphocyte infiltration in various types of human cancers (Robinson et al., 2003, Soria and Ben-Baruch, 2008). In a transplantable model of breast carcinoma, CCL5 was determined to be expressed by tumor cells, while its receptor, CCR5, was localized to infiltrating macrophages and lymphocytes. Furthermore, the dual CCRI/CCR5 antagonist, Met-CCL5, was able to reduce tumor growth and inhibit the migration of macrophages and lymphocytes into 410.4 tumors, suggesting a potential role of CCL5 in tumor-promoting macrophage/lymphocyte infiltration (Robinson et al., 2003). A query of the NCI REMBRANDT (National Cancer Institution REpository for Molecular BRAin Neoplasia DaTa) database using CCL5 as the search term, revealed an association between CCL5

expression and survival outcomes in “All Glioma” patients. A significantly shorter mean survival time was evident in tumors having high CCL5 as compared to those characterized by intermediate levels of CCL5; insufficient numbers of patients with low CCL5 limited analysis of this patient population. The observations suggest a significant role of CCL5 within glioma, as well as a potential mechanism of immune and tumor cells interaction, through this ligand.

Although the expression of CCL5 and its receptors, CCR1 and CCR5, is documented in different cancer models, no study on the role of the CCL5/CCR1/CCR5 axis in glioblastoma has been reported. Herein, we establish that CCL5 is highly expressed in murine and human GBM cell lines, and found abundantly in tumors of glioblastoma bearing mice where CCR1 and CCR5 are also expressed. Individual deletion of either CCR1 or CCR5 had little to no impact on survival rates of glioblastoma-bearing or the numbers of tumor-infiltrated immune CD11b⁺, CD4⁺, and CD8⁺ cells. An *in vitro* analysis showed that CCR1 and CCR5 were expressed by primary microglia suggesting functional redundancy in this system. Indeed, the dual CCR1/CCR5 antagonist, Met-CCL5, was able to inhibit CCL5-dependent migration of wild type, CCR1- and CCR5-deficient microglia. Collectively, the results indicate that the infiltration of microglia/macrophages into glioblastoma, as well as the survival of tumor bearing mice, does not solely depend on either CCR1 or CCR5 but suggests a potential mechanism of redundancy, where CCL5 directs the infiltration of microglia/macrophages into glioblastoma through both CCR1 and CCR5.

2. Materials and Methods

2.1. Animals

Wild type (WT) C57BL/6 mice were obtained from either JAX Laboratory (Bar Harbor, ME) or Taconic Inc. (Hudson, NY). CCR1- and CCR5-deficient mice, backcrossed to the C57BL/6 background for greater than 10 generations, were purchased from Taconic Inc. and JAX Laboratory, respectively. Experimental endpoints from CCR1-deficient mice were compared to WT mice purchased from Taconic Inc. while endpoints from CCR5-deficient mice were compared to WT mice obtained from the JAX Laboratory. All procedures involving animal housing and surgical protocols were followed according to the guidelines of the University of Florida Institutional Animal Care and Use Committee (IACUC).

2.2. Cell culture

The human glioblastoma cell lines T98G and U87 were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% sodium pyruvate and 2mM L-glutamine. The U118 GBM cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 2mM L-glutamine. Primary patient derived GBM cell lines L0, L1, and L2, generously provided by Dr. Brent A. Reynolds (Department of Neurosurgery, University of Florida), were cultured in DMEM/F12 medium supplemented with 2% B27, 20ng/ml of epidermal growth factor (EGF) and 1% penicillin-streptomycin. The GL261 murine glioblastoma cell line was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, and 4mM L-glutamine. To generate gliomaspheres, GL261 glioblastoma cells were cultured in DMEM/F12 medium supplemented with 2% B27, 20ng/ml of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), 5 µg/ml of heparin and 1% penicillin-streptomycin, using non Poly-D-Lysine treated T75 flasks. All cells were grown in a humidified incubator at 37°C with 5% CO₂. DMEM, EMEM, RPMI-1640, DMEM/F12 medium, B27, EGF, bFGF, L-glutamine and antibiotics were obtained from Gibco-BRL (Invitrogen, Carlsbad, CA). Sodium pyruvate and heparin were

purchased from Sigma-Aldrich (St Louis, MO). FBS was from HyClone (Thermo Scientific, Waltham, MA)

2.3. Primary microglia isolation

Primary microglia were harvested from postnatal one day old mouse pups using a previously published protocol (Saura et al., 2003). Briefly, brain tissue was removed, mechanically and enzymatically dissociated, and kept in medium A containing 0.585% glucose (Sigma-Aldrich), 15mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin in HBSS (Gibco). The finely minced brain tissue was incubated in 0.25% trypsin medium (Gibco) for 30–45 minutes at 37°C. The medium was aspirated and replaced with trypsin inhibitor medium (Invitrogen). After incubation for 4 min at room temperature, the tissue was triturated with a fire-polished glass pipette and then centrifuged for 15 min at 100×g. The supernatant was aspirated and the cell suspension was plated in T75 flasks with DMEM/F12 medium supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin and streptomycin. Culture medium was changed every 3–4 days. After 15 days, cultures were treated with 0.0625% trypsin-EDTA (diluted in DMEM/F12) for 1 hour at 37°C to lift astrocytes and neurons from the flasks, leaving an essentially pure culture of primary microglia. The cultures were checked for purity and found to be greater than 97% microglia as measured by cell-type specific expression of CD11b. Purified primary microglia were collected using 2.5% trypsin with EDTA for RT-PCR and migration analyses.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

To isolate total RNA from normal brain and tumor tissues, approximately 1cm³ fragments of normal and tumor tissues were removed from the brains of GL261-implanted mice and immersed immediately in TRIzol reagent (Invitrogen). The normal tissue was collected from the non-implanted hemisphere to avoid contamination of tumor tissue. All samples were homogenized with a homogenizer and processed to RNA isolation as indicated by the manufacturer's instructions. Total RNA was also isolated from microglia and glioma cells with the TRIzol reagent. Genomic DNA contamination was removed by RQ1 RNase-free DNase treatment (Promega, Madison, WI). Total RNA was then quantified and stored at –80°C. RNA (1µg) was retrotranscribed with iScript complementary DNA (cDNA) synthesis kit (BioRad, Hercules, CA). Synthesized cDNA was subjected to PCR analysis. PCR was performed by heating for 96°C for 2 min, followed by amplification for 35 cycles: 96°C for 30 sec, 56°C for 1 min, and 72°C for 1 min. The following primers were used: murine CCL5: 5' ggtaccatgaagatctctgca 3' (forward) and 5' agcaagccatgacaggaagc 3' (reverse); human CCL5: 5' cgtgccacatcaaggag 3' (forward) and 5' ggacaagacaagcagaac 3' (reverse); murine CCR1: 5' gtggtggcaatgtcctagt (forward) and 5' tcagattgtaggggtccag 3' (reverse); murine CCR5: 5' tcagttccgacctatctatg 3' (forward) and 5' gtggaaatgaggactgcatgt 3' (reverse); murine CX3CR1: 5' atgcatgtgcaagctca 3' (forward) and 5' cttcatgtcacaactggg 3' (reverse); murine glyceraldehyde 3-phosphate dehydrogenase: 5' aaatggtgaaggtcggtgtg 3' (forward) and 5' tctccatggtggaagaca 3' (reverse); human glyceraldehyde 3-phosphate dehydrogenase: 5' cgagatccctcaaatcaa 3' (forward) and 5' tgctgtagccaatctgtg 3' (reverse). The RT-PCR analyses on tumor and normal brain tissues were repeated two times using two different animals. With primary cultured microglia, the experiments were independently performed at least three times with different microglia preparations.

2.5. Cytokine protein array

The presence of cytokines in GL261 cell conditioned medium was determined by mouse cytokine antibody array (R&D System, Minneapolis, MN). Membranes were treated with blocking buffer and incubated for 30 min at room temperature. The membrane was then exposed to an aliquot (1 ml) of GL261 conditioned medium and incubated for 1 h at room

temperature. After this incubation period, the membranes were washed five times with a washing buffer and incubated for 1 h at room temperature with biotin-conjugated antibodies against murine cytokines (dilution: 1:250 dilution). Thereafter, the membranes were washed five times, incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated streptavidin (dilution: 1:1000) and washed five times again. Finally, the reaction was developed in a mixture of SuperSignal West Pico luminol/enhancer and stable peroxide solutions (Thermo Scientific, Rockford, IL) and exposed to X-ray film. The densities of signals on films were then analyzed with ImageJ software (NIH). This experiment was performed in duplicate.

2.6. Glioma cell implantations

GL261 glioma cells (1×10^5) in a total volume not exceeding 3 μ l were injected 3mm deep into the right cerebral hemisphere (1mm posterior and 2mm lateral from Bregma) of wild type, CCR1-, and CCR5-deficient mice. For Kaplan-Meier survival rate analysis, percentages of surviving mice in the various groups of animals were recorded daily after GL261 glioma implantation. The endpoint was defined by a lack of physical activity and a body weight reduction of greater than 15%. The data were subjected to log-rank test in order to determine if significant differences existed in survival between the experimental groups. The numbers of animals used in each group are indicated in the graphs. For other experiments, glioma bearing mice (3 weeks after GL261 cell injection) were euthanized using sodium pentobarbital (32mg/kg) and subsequently perfused with 0.9% saline followed by buffered 4% paraformaldehyde (PFA). Brains were surgically removed and post-fixed with 4% PFA. After fixation, tissues were incubated in 30% sucrose solution at 4°C overnight followed by liquid nitrogen freezing. Frozen brains were then sectioned with a cryostat and subjected to either *in situ* hybridization or immunohistochemistry.

2.7. *In situ* hybridization and immunohistochemistry

In situ hybridization was performed as described previously (Harrison et al., 2003). Multiple sections from tumor bearing animals were analyzed and included the following number of mice: wild type (JAX Laboratory): 11; wild type (Taconic Inc): 9; CCR1^{-/-}: 7; CCR5^{-/-}: 2. For immunohistochemistry, brain sections were permeabilized with 0.5% of Triton X-100 in phosphate-buffered saline (PBS) for 15 min at room temperature followed by blocking with 10% goat serum in PBS for 30 min. The sections were then incubated in primary antibodies at room temperature. The following antibodies were used: rat anti-CD4 (dilution 1:50, BD Pharmingen), rat anti-CD8 (dilution 1:50, Serotec), rat anti-CD11b (dilution 1:50, Serotec). After 2 hours, sections were washed three times with PBS and incubated subsequently in goat anti-rat Alexa 594 (dilution 1:1000, BD Pharmingen). The sections were then washed three times with PBS and finally counterstained with DAPI. For quantification of CD4⁺, CD8⁺, and CD11b⁺ cells, the number of cells in three high-powered field, which was the visible area under 20 \times magnification, in three sections from multiple animals were calculated to determine the mean and standard error of mean. The data were subjected to statistical analysis (one tail T-test). The numbers of animals used in each group are indicated in the graphs.

2.8. *In vitro* migration

In vitro migration assays were performed using 24-well transwell units with 8- μ m polycarbonated filters (BD Falcon, Franklin Lakes, NJ). After trypsinization with 2.5% trypsin, microglia (2×10^4 cells) were placed in the top compartment in 500 μ l of serum-free DMEM/F12 with or without 50nM of Met-CCL5. The bottom wells contained 500 μ l of serum-free DMEM/F12 with or without 20nM of CCL5. The plates were incubated for 24 hours at 37°C. Non-migrating cells were removed by wiping the upper side of the insert with a cotton swab. Migrating cells were fixed with 4% paraformaldehyde for 20 min at room

temperature, stained with hematoxylin, and quantified by counting the number of cells on 4 random areas on the lower side of the membrane. Each experiment was performed in triplicate and repeated three times using different microglial preparations.

2.9. Statistical analysis

All statistical analyses were calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). All data are presented as mean and standard error of mean. P-values were calculated using Student's t-test with two-tailed distribution. Survival data were subjected to log-rank test to determine statistically significant differences between groups. A p-value <0.05 was considered significant and is indicated by symbols depicted in the figures and figure legends.

3. Results

3.1. CCL5 is expressed by murine GL261 and human GBM cell lines

Because chemokine CCL5 has been found in various types of tumor, including prostate, cervical, and breast cancers (Vaday et al., 2006, Niwa et al., 2001), we were interested in determining if CCL5 was also present in glioblastoma. The expression of CCL5 *in vitro* was assessed by RT-PCR analysis in the murine GL261 glioblastoma cell line. The results showed that CCL5 mRNA was present in cells cultured as adherent monolayers and under culture conditions that favored formation of gliomaspheres; increased levels of CCL5 mRNA were noted in the gliomaspheres. In addition, CCL5 peptide was detected in the GL261 conditioned medium, using a cytokine protein array (Fig. 1A), indicating that GL261 cells are capable of both producing and releasing CCL5 protein. The expression of this chemokine was also identified in several different human glioblastoma cells. CCL5 mRNA, detected by RT-PCR analysis, was found in primary patient-derived lines L0, L1, and L2 that were cultured under serum-free conditions in the presence of EGF and bFGF, as well as the commonly used glioblastoma lines T98G, U87, and U118 cultured in the presence of serum (Fig. 1B). None of the lines expressed CCR1 and CCR5 (data not shown).

3.2. CCL5, CCR1, and CCR5 are present in murine GL261 glioblastomas

The *in vivo* expression of CCL5 in the tissues taken from GL261 glioblastoma-bearing wild type mice was evaluated by RT-PCR and *in situ* hybridization analyses. In both cases, mRNA levels of CCL5 were significantly elevated in the tumor tissue, when compared to the normal brain area (Fig. 2A, B). Together with the *in vitro* data above, these results suggested that tumor cells are the primary source for CCL5 production within glioblastoma. In addition, the two major receptors of this chemokine, CCR1 and CCR5, were strongly detected in the tumor tissues (Fig. 2A, C). We also evaluated the expression of CCR3, another low affinity CCL5 receptor. The lack of signal of this receptor from *in situ* hybridization analysis indicated that CCR3 was not expressed in intracranial GL261 glioblastomas (Fig. 2C). Immunohistochemical localization of CCR1 and CCR5 within the tumors was evaluated but the lack of availability of suitable antibodies precluded definitive identification of these receptors.

The expression of CCL5 and its receptors, CCR1 and CCR5, was also analyzed in the tissues collected from tumor bearing CCR1^{-/-} and CCR5^{-/-} mice. *In situ* hybridization analysis showed that CCL5 was expressed at high levels within tumors of these animals, suggesting that CCR1 and CCR5 deficiency did not affect the expression of this chemokine within the tumor. In contrast, while both CCR1 and CCR5 were present in the tumors from wild type mice, the signals for CCR1 and CCR5 were greatly diminished in glioblastomas from CCR1^{-/-} and CCR5^{-/-} mice, respectively (Fig. 2C). This observation indicates that

intratumoral CCR1- and CCR5-expressing cells are derived from the recipient mice, and not from the culture-derived GL261 glioblastoma cells that were implanted into the animals.

3.3. Neither CCR1 nor CCR5 deficiency contributed to the infiltration of immune cells into glioblastoma, or impacted the survival of tumor bearing mice

To evaluate the hypothesis that CCL5 directs the localization of microglia/macrophages into glioblastoma in a CCR1 or CCR5 dependent manner, we characterized immune cell infiltration and animal survival in tumor bearing CCR1- and CCR5-deficient mice. Immunohistochemical analysis was performed to investigate if CCR1 and CCR5 regulated the infiltration of immune cells, including CD11b⁺ microglia/macrophages, CD4⁺ and CD8⁺ T cells, into glioblastoma. Quantitative analysis did not show any statistically significant differences in the number of these cells located within the glioblastomas of tumor bearing CCR1^{-/-} and CCR5^{-/-} mice, as compared to the wild type animals (Fig. 3A); the supplementary figure depicts representative images of the immunohistochemistry. The effect of CCR1 and CCR5 deficiency on the survival of glioblastoma bearing mice among wild type, CCR1^{-/-}, and CCR5^{-/-} groups was also assessed using Kaplan-Meier survival analysis. No significant difference in the survival rate between tumor bearing wild type and CCR5^{-/-} groups were evident (p value = 0.77). The life span of glioblastoma bearing CCR1^{-/-} mice, however, was slightly shorter than the life span of the wild type (p value = 0.0007). The median survival time of tumor bearing CCR1^{-/-} mice after GL261 cell implantation was 19 days, while that of glioblastoma bearing wild type mice was 22 days. Together, these results suggest that CCR1 and CCR5 deficiency had no impact on the infiltration of CD11⁺, CD4⁺, and CD8⁺ immune cells into glioblastoma, and at best, only a modest effect on the survival rate of tumor bearing mice.

3.4. CCL5 interacts with its microglia-expressed receptors, CCR1 and CCR5, in a redundant manner

As neither CCR1 nor CCR5 contribute individually to the action of CCL5 on the localization of microglia/macrophages into glioblastoma, as well as the survival of tumor bearing mice, we next examined the possibility that CCL5 may interact with CCR1 and CCR5 in a redundant manner. The *in vitro* expression of CCR1 and CCR5 was determined on primary cultures of microglia derived from wild type, CCR1^{-/-}, and CCR5^{-/-} mice. Both CCR1 and CCR5 mRNAs were detected in microglia using RT-PCR analysis, suggesting that these CCL5 receptors are co-expressed by these cells *in vitro* (Fig. 4A). The absence of CCR1 and CCR5 mRNA signals in CCR1^{-/-} and CCR5^{-/-} mice, respectively, confirmed that these gene products were deleted in the microglia derived from the CCR1- and CCR5-deficient animals. CX3CR1, a microglia-expressed chemokine receptor, was detected in microglia cultures from all of the animals. We also pursued the expression of CCR1 and CCR5 protein by microglia *in vitro*, but similar to our attempts to detect these receptors *in vivo*, the lack of quality antibodies reactive towards murine CCR1 and CCR5 posed significant challenges to identifying these receptors. Thus, a functional analysis of CCL5-dependent migration of microglia was assessed using the modified Boyden chamber assay (Fig. 4B). Migration of wild type, as well as CCR1^{-/-} and CCR5^{-/-} microglia was stimulated by CCL5. Met-CCL5, a modified form of CCL5 that has antagonist activity at both CCR1 and CCR5, completely blocked the migration by CCL5 in all groups.

4. Discussion

The presence of a high number of infiltrated microglia/macrophages within glioma, confirmed by various studies both in human glioma and rodent models of the disease (Graeber et al., 2002, Liu et al., 2009, Charles et al., 2011), has prompted further investigations into the role of these immune competent cells within the tumor

microenvironment. Accumulating evidence suggests that microglia/macrophages facilitate glioma growth by contributing to the immunosuppressive environment and directly assisting in tumor growth and invasion (Markovic et al., 2005, Markovic et al., 2009, Sliwa et al., 2007, Morimura et al., 1990, Zhai et al., 2011). Understanding the mechanisms by which microglia/macrophages cells are localized to glioma will provide novel therapeutic targets for intervention. Here we report on the expression of CCL5 and its receptors, CCR1 and CCR5, in glioblastoma. Our data demonstrate that CCL5 is highly expressed by human and murine GL261 GBM cells *in vitro*, as well within intracranial glioblastomas *in vivo*. Moreover, CCL5 is upregulated in GL261 cells cultured under condition that favors growth of the cells as spheres; cells cultured under this condition exhibit a more malignant phenotype (Lee et al., 2006, Pellegatta et al., 2006). Its receptors CCR1 and CCR5, on the other hand, are expressed by cultured microglia, but not by glioblastoma cells, and are also highly expressed within intracranial tumors. The loss of CCR1 and CCR5 *in situ* hybridization signals from tumor sections from CCR1- and CCR5-deficient mice also indicates that the glioblastoma cells do not express either of these receptors. These data support the hypothesis that glioblastoma-expressed CCL5 may be a key regulator in the crosstalk between glioblastoma and microglia/macrophages, through an interaction with microglia/macrophages-expressed CCR1 or CCR5, and that blocking either CCR1 or CCR5 could prevent the glioblastoma localization of microglia/macrophages.

Glioblastoma growth and numbers of intra-tumoral microglia/macrophages cells, together with CD4⁺ and CD8⁺ T cells, were evaluated in mice individually deficient in either CCR1 or CCR5. Our data showed that microglia/macrophages infiltration was not attenuated in either CCR1- or CCR5-deficient tumor-bearing animals. CD4⁺ and CD8⁺ cells were also similar in tumors from receptor deficient and wild type mice. Moreover, there was only a modest, in the case of CCR1-deficiency, or no difference, with CCR5-deficiency, in survival rates between glioblastoma-bearing wild type mice and the chemokine receptor-deficient tumor-bearing animals. Our explanation for these observations is that CCL5 has a high affinity for both CCR1 and CCR5, and blocking either one of them individually is not sufficient to inhibit the actions of this chemokine. Due to the promiscuous interactions of chemokines and chemokine receptors, this redundant mechanism needs to be considered. Indeed, this issue has been addressed in several studies (Iida et al., 2008, de Lemos et al., 2005, Remick et al., 2001, Repeke et al., 2010). In a recent study that characterized the role of these receptors in a periodontal disease model, Repeke and colleagues found that CCR1- and CCR5-deficient mice present a lower leukocyte infiltration and alveolar bone loss than wild type mice, yet inhibiting both of these receptors by Met-CCL5 (a dual CCR1/CCR5 antagonist) severely attenuated the inflammatory bone resorption (Repeke et al., 2010). A redundancy mechanism in glioblastoma is consistent with our *in vitro* investigation which indicates that CCR1 and CCR5 are co-expressed by primary microglia *in vitro*, and that the dual antagonist Met-CCL5 could completely block the migratory effect of CCL5 on these cells regardless of whether the cells expressed CCR1 or CCR5. Nonetheless, our data does not exclude the possibility that CCL5 may act through another unidentified receptor(s).

While the evidence for a cooperative role of CCR1 and CCR5 is suggested from the *in vitro* study, a redundancy of these chemokine receptors in this murine glioblastoma model has not yet been evaluated *in vivo*. A combined CCR1- and CCR5-deficient mouse would offer a model system to test the redundancy hypothesis. However, the CCR1 and CCR5 genes are located adjacent to each other on murine chromosome 9, providing a major limitation in generating the double knockout mice by simply breeding CCR1- and CCR5-deficient lines. Alternative pharmacologically-based approaches are worth considering but these also have limitations. Although Met-CCL5 has been used as a dual antagonist for both CCR1 and CCR5 in *in vitro* studies (Proudfoot et al., 1996, Robinson et al., 2003), effectively targeting these receptors located within the brain would require the peptide to penetrate the blood

brain barrier, one of the most important obstacles that restricted the delivery of molecularly targeted therapy to glioma (Agarwal et al., 2011). A combined pharmacological and genetic approach is an alternative strategy worthy of consideration. In particular, CCR1-deficient mice could be treated with a CCR5 antagonist, e.g. TAK-779, while CCR5-deficient mice would be treated with the CCR1 antagonist, BX-741. Both of these drugs have been tested for their effective delivery to the CNS, when administered systemically (Liang et al., 2000, Longden et al., 2008, Maeda et al., 2006, Ni et al., 2009). However, as BX-741 and TAK-779 were specifically designed for human CCR1 and CCR5 respectively, the low binding affinity for mouse CCR1 and CCR5 is a primary concern. These obstacles together provide significant challenges for addressing the redundant hypothesis through *in vivo* experimentation.

The interaction between microglia/macrophages and glioblastoma is complex and likely to involve bi-directional signaling. After recruitment into the tumor milieu by glioblastoma cells, microglia/macrophages lose control of their immune surveillance property and, in turn, help facilitate tumor progression. Since the recruitment of microglia/macrophages likely depends on their interaction with numerous glioblastoma-secreted factors or by other components in the tumor microenvironment, it is not surprising that this process is regulated in a redundant manner. Our findings support the notion that the function of CCL5 in glioblastoma is not dependent on individual interactions with either CCR1 or CCR5. To the contrary, the CCL5/CCR1/CCR5 network likely involves a redundant mechanism, adding more complexity to glioblastoma-microglia/macrophages crosstalk and signaling a need to block both receptors to prevent the actions of this chemokine in this highly malignant form of brain cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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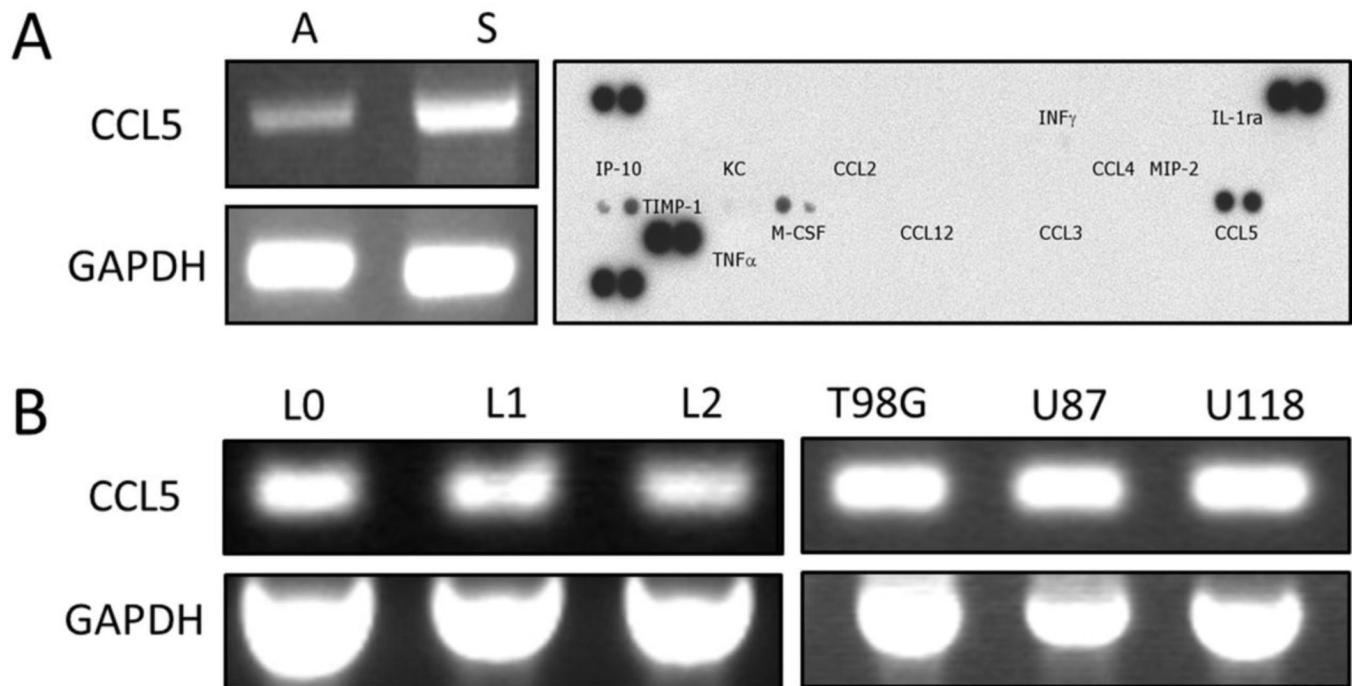


Figure 1.

Expression of CCL5 by murine GL261 and human GBM cell lines. A) Left: RT-PCR analysis detecting CCL5 mRNA in murine GL261 cells, cultured as either adherent monolayers (A) or gliospheres (S). GAPDH served as a control. Right: CCL5 protein detected in GL261 conditioned medium, using cytokine protein array. B) RT-PCR analysis of CCL5 mRNA in different human GBM cell lines, L0, L1, and L2 as well as commonly used standard glioblastoma cell lines, T98G, U87, and U118. GAPDH served as a control.

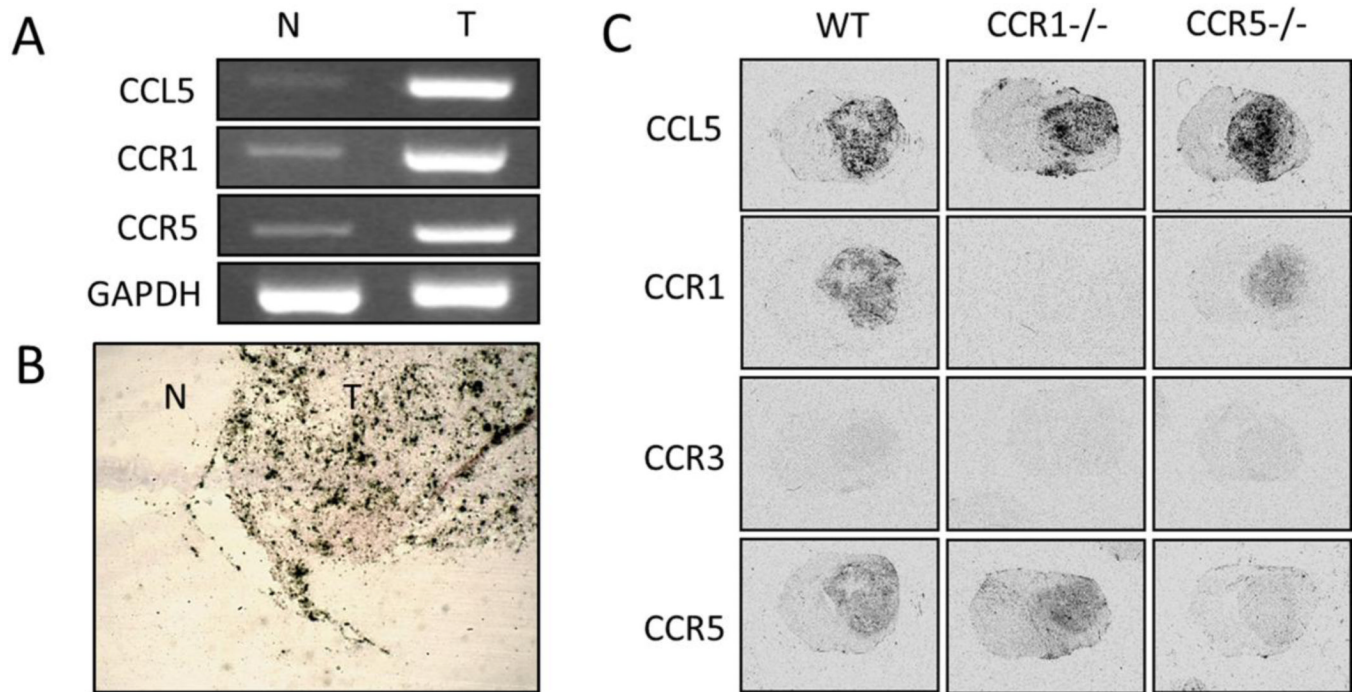


Figure 2. Expression of CCL5 and its receptors CCR1 and CCR5 in murine GL261 glioblastomas. A) RT-PCR analysis in normal (N) and tumor (T) tissues. In situ hybridization (ISH) analysis depicting the expression of B) CCL5 in a section containing normal (N) and tumor (T) tissue from a glioma-bearing wild type (WT) C57BL/6 mouse, and C) CCL5, CCR1, CCR3, and CCR5 in WT, CCR1 and CCR5-deficient mice.

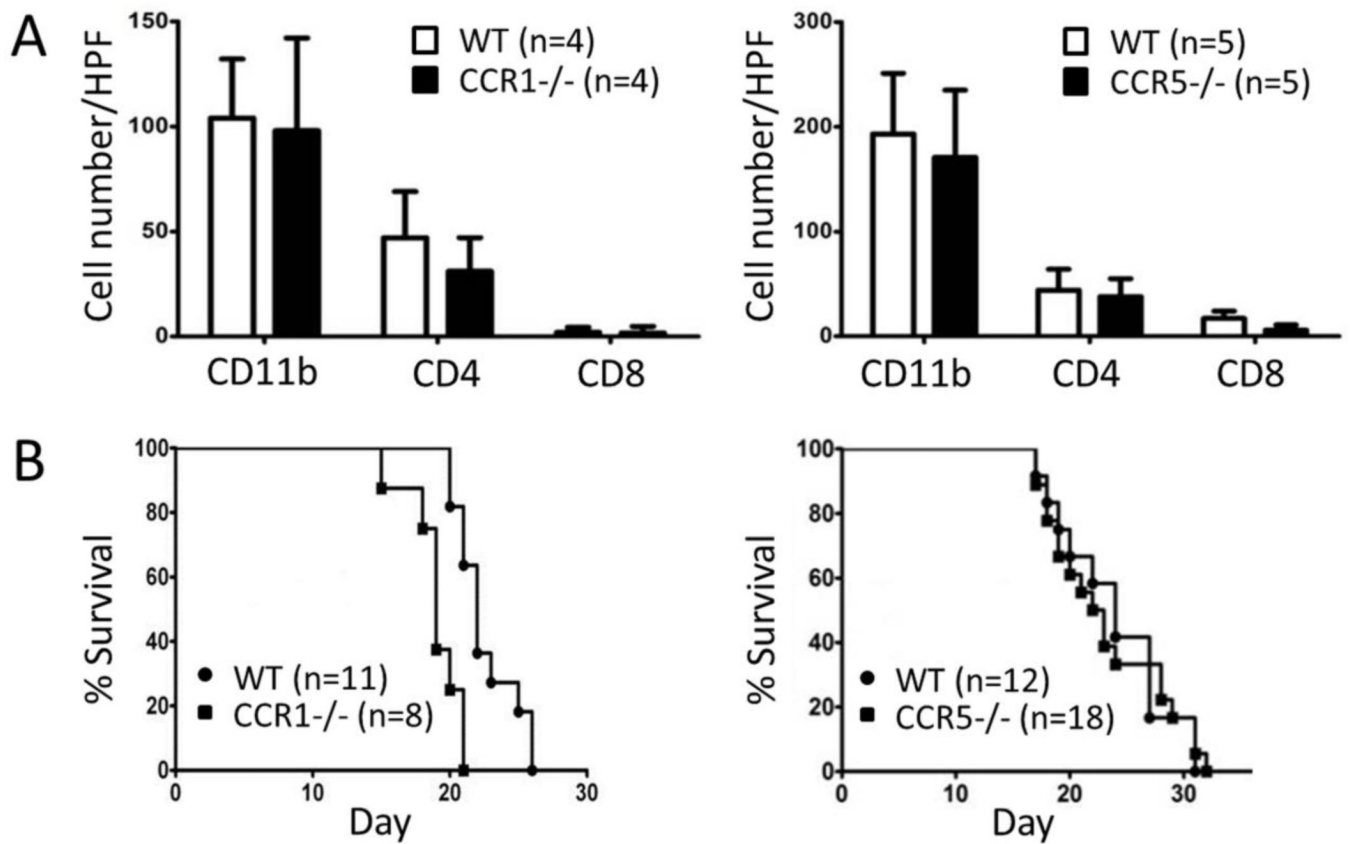


Figure 3. Effect of CCR1 and CCR5 deficiency on infiltration of immune cells into glioblastoma and survival of tumor bearing mice. A) Numbers of intra-glioblastoma CD11b⁺, CD4⁺, and CD8⁺ in CCR1 (left, black) and CCR5 (right, black)-deficient mice, compared to WT (white) mice. B) Kaplan-Meier survival plots of glioblastoma bearing CCR1 (left, square) and CCR5 (right, square)-deficient mice, compared to WT (circles) mice. The numbers of animals that were used in each group are indicated.

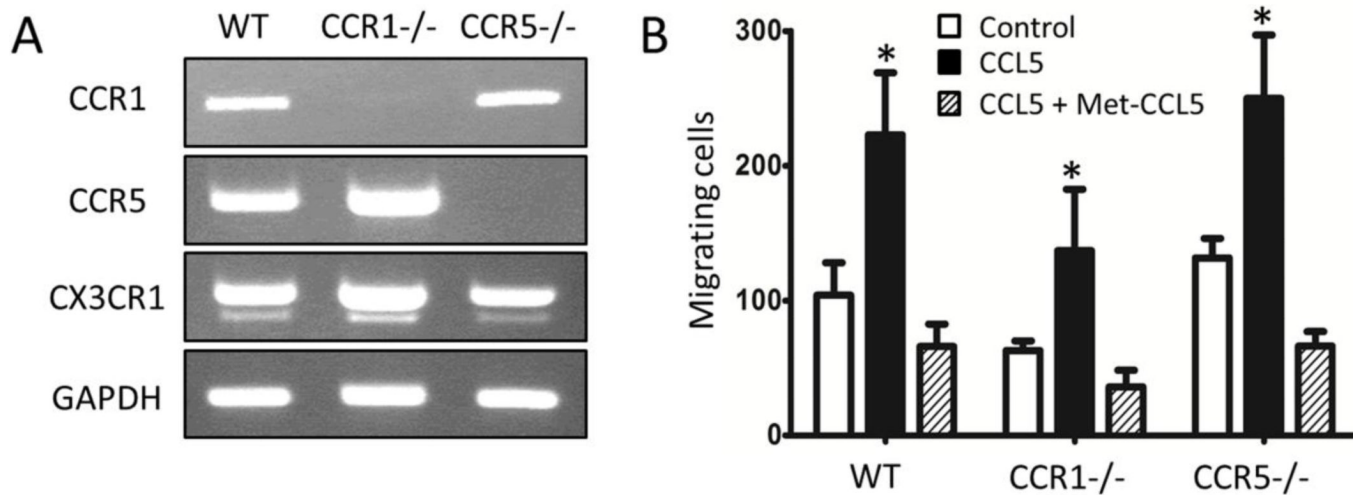


Figure 4.

In vitro expression of CCR1 and CCR5 by primary microglia cultures and the effect of Met-CCL5 on CCL5-stimulated migration of microglia. A) RT-PCR analysis of CCR1 and CCR5 expressed by WT, CCR1- (CCR1^{-/-}), and CCR5- (CCR5^{-/-}) deficient microglia. CX3CR1 and GAPDH served as controls. B) Migration of WT, CCR1-, and CCR5-deficient microglia treated with CCL5 (black), or with CCL5 and Met-CCL5 (stripe), compared to control (white). * $p < 0.05$, compared to either control or CCL5/Met-CCL5.