Recruitment of CCR2\(^+\) tumor associated macrophage to sites of liver metastasis confers a poor prognosis in human colorectal cancer

Julie G Grossman\(^{a}\), Timothy M Nywening\(^{b}\), Brian A Belt\(^{c,e,f}\), Roheena Z Panni\(^{a}\), Bradley A Krasnick\(^{a}\), David G DeNardo\(^{b,c,d}\), William G Hawkins\(^{a,d}\), S Peter Goedegebuure\(^{a,d}\), David C Linehan\(^{e,f,g}\), and Ryan C Fields\(^{a,d}\)

\(^{a}\)Department of Surgery, Washington University School of Medicine, St. Louis, MO, USA; \(^{b}\)Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA; \(^{c}\)Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA; \(^{d}\)Alvin J Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO, USA; \(^{e}\)Department of Surgery, University of Rochester Medical Center, Rochester, NY, USA; \(^{f}\)Tumor Immunology Program, University of Rochester Medical Center, Rochester, NY, USA; \(^{g}\)Wilmot Cancer Institute, University of Rochester Medical Center, Rochester, NY, USA

ABSTRACT

The tumor microenvironment (TME) represents a significant barrier to creating effective therapies for metastatic colorectal cancer (mCRC). In several malignancies, bone marrow derived CCR2\(^+\) inflammatory monocytes (IM) are recruited to the TME by neoplastic cells, where they become immunosuppressive tumor associated macrophages (TAM). Here we report that mCRC expression of the chemokine CCL2 facilitates recruitment of CCR2\(^+\) IM from the bone marrow to the peripheral blood. Immune monitoring of circulating monocytes in patients with mCRC found this influx was a prognostic biomarker and correlated with worse clinical outcomes. At the metastatic site, mCRC liver tumors were heavily infiltrated by TAM, which displayed a robust ability to dampen endogenous anti-tumor lymphocyte activity. Using a murine model of mCRC that recapitulates these findings from human disease, we show that targeting CCR2 reduces TAM accumulation in liver metastasis and restores anti-tumor immunity. Additional quantitative analysis of hepatic metastatic tumor burden and treatment efficacy found that administration of a small molecule CCR2 inhibitor (CCR2\(^i\)) improves chemotherapeutic responses and increases overall survival in mice with mCRC liver tumors. Our study suggests that targeting the CCL2/CCR2 chemokine axis decreases TAM at the metastatic site, disrupting the immunosuppressive TME and rendering mCRC susceptible to anti-tumor T-cell responses.

Introduction

Colorectal cancer (CRC) is the second most lethal malignancy in the US and accounted for more than 50,000 cancer related deaths in 2016.\(^1\) Approximately 25% of patients will present with synchronous metastatic colorectal cancer (mCRC), most commonly to the liver, at the time of diagnosis and while hepatic resection can improve survival, 60% of patients develop disease recurrence within 2 years.\(^2,3\) Frontline chemotherapy, consisting of a 5-fluorouracil-based treatment combined with either oxaliplatin or irinotecan (FOLFOX or FOLFIRI, respectively) has improved survival for patients with inoperable advanced mCRC and may provide benefit as neoadjuvant therapy in patients with resectable metastatic disease.\(^4,5\) However, despite these advances the majority of patients with mCRC will ultimately succumb to their disease and novel therapies are urgently needed.

The presence of effector tumor infiltrating lymphocytes (TIL), including cytolytic CD8\(^+\) and Th1 CD4\(^+\) T-cells, has been used to establish an immunoscore correlating with favorable clinical outcomes in mCRC.\(^6,7\) Recently, checkpoint targeted therapies have shown promise in mCRC patients with high mutational loads, generally attributable to mutations in the DNA mismatch repair genes (e.g. MLH1 and Lynch Syndrome), demonstrating the potential for immune modulating therapies in mCRC.\(^8\) However, there has been little emphasis placed on the role of myeloid cells in the tumor microenvironment (TME) that limit the capacity of the adaptive immune system to mount a robust anti-tumor response.\(^9\)

Cancer promoting inflammation orchestrates an intense immune cell response and the TME is emerging as a critical barrier to the development of effective therapies.\(^10–12\) In particular, tumor associated macrophages (TAM) are a prominent myeloid population in the TME and have been implicated in neoplastic progression, avoidance of immune detection, and metastatic dissemination in a variety of solid tumors.\(^13,14\) In humans, the CCL2/CCR2 chemokine axis has been implicated in cancer mediated mobilization of inflammatory monocytes (IM) from the bone marrow by tumor expression of the chemokine CCL2.\(^15–17\) Circulating IM (CD11b\(^+\), CD14\(^+\), CCR2\(^+\)) ultimately migrate to malignant sites, where they become immunosuppressive TAM (CD11b\(^+\), CD14\(^+\), CD68\(^+\)) and...
promote tumor progression. Similarly, tumor bearing mice recruit IM (CD11b\(^+\), Ly6C\(^{hi}\), CCR2\(^+\)) with a heavy TAM (CD11b\(^+\), Gr1\(^{lo}\), F4/80\(^{hi}\)) infiltrate found at primary and metastatic sites.\(^{15,18,19}\) Targeting the CCL2/CCR2 pathway has shown promise in preclinical studies and results from a clinical trial reported by our group demonstrated promising therapeutic responses following treatment with a small molecule CCR2 inhibitor (CCR2i) in patients with borderline resectable and locally advanced pancreatic adenocarcinoma.\(^{18–21}\) Based on this evidence, we hypothesized that CCR2 blockade may represent a promising treatment strategy in metastatic disease and thus sought to further investigate using a preclinical model that recapitulated findings from clinical correlative studies of patients with mCRC.

Here we report that the CCL2/CCR2 chemokine axis recruits bone marrow derived TAM to metastatic sites in CRC patients. Therapeutic targeting of this pathway via CCR2 blockade reduces TAM which improved chemotherapeutic responses in preclinical studies using a murine model of mCRC. In summary, these findings support therapeutic targeting of CCR2\(^+\) TAM recruitment as an emerging treatment modality in mCRC.

**Results**

**Preoperative monocyte percentage correlates with poor prognosis in patients with colorectal liver metastasis**

Using a prospectively maintained database of patients undergoing hepatic resection for mCRC (n = 221), subjects were stratified and compared according to the median preoperative percentage monocytes on peripheral complete blood counts (CBC) into high (≥8.9%), and low (<8.9%) groups. Baseline patient demographics and tumor characteristics are included in the supplemental materials (Supplemental Table S1). Overall, an elevated preoperative monocyte level was associated with early disease recurrence (Figure 1A). Patients in the high monocyte cohort (n = 111) had a significantly shorter

![Figure 1](https://example.com/figure1.png)

**Figure 1. Elevated circulating CCR2\(^+\) inflammatory monocytes correlate with poor prognosis in mCRC patients.** (A) Patients with hepatic mCRC were stratified into high (≥8.9%; n = 111) and low (<8.9%; n = 110) cohorts based on percentage of monocytes in the peripheral blood obtained from preoperative CBC within 30 days of index operation and analyzed for recurrence-free survival following liver resection for mCRC. (B) High and low preoperative monocyte cohorts analyzed for overall survival following index hepatic resection of mCRC. (C) Left panels, representative flow cytometry plots of inflammatory monocytes (CD11b\(^+\), CD14\(^+\), CCR2\(^+\)) from the peripheral blood of healthy controls (Top) and mCRC patients (Bottom). Right panel, graph depicts inflammatory monocytes as percentage of CD45\(^+\) cells by flow cytometry analysis in healthy controls (n = 16) and mCRC patients (n = 30). (D) Comparison of inflammatory monocytes as a percentage of CD45\(^+\) cells by flow cytometry analysis between mCRC patients surviving greater (n = 20) or less than (n = 10) 1-year following mCRC liver resection. Comparison between groups using a two-tailed unpaired t-test or Kaplan-Meier survival analysis used to calculate p-values and shown as mean ± SD.
median recurrence-free survival of 13.1 months compared to 18 months in the low monocyte group (n = 110) following liver resection for mCRC (HR = 1.50 [95% CI: 1.09–2.22]). Likewise, a high preoperative monocyte count was associated with a worse prognosis and reduced overall survival following liver resection for mCRC (Figure 1B). Patients with a high preoperative monocyte count had a median survival of 2.72 years, which was significantly less than the median survival of 4.85 years in the low monocyte group (HR = 2.1 [95% CI: 1.53–3.02]). Using multivariate analysis, elevated preoperative monocyte percentage was found to be an independent predictor of worse prognosis following hepatectomy for mCRC with a 5-year survival of 21.59% vs 46.9% in the high and low monocyte groups respectively (Supplemental Table S2). In summary, this data supports peripheral blood monocyte counts as a prognostic indicator in mCRC.

**CCR2⁺ inflammatory monocytes are elevated in human mCRC and associated with shorter survival following liver resection**

As tumor associated macrophages (TAM) are derived from CCR2⁺ inflammatory monocytes (IM) we analyzed peripheral blood specimens by flow cytometry to ascertain if the CCR2⁺ IM population in patients with mCRC could account for the increase in monocyte percentage found on routine preoperative laboratory assessment. This demonstrated that circulating CCR2⁺ IM are elevated in human mCRC patients compared to healthy controls (Figure 1C). Furthermore, the percentage of CCR2⁺ IM in the peripheral blood was associated with worse clinical outcomes, with patients surviving less than 1-year following mCRC liver resection having significantly higher CCR2⁺ IM in the blood compared to those surviving greater than 1-year (Figure 1D). Collectively, this data supports our hypothesis that circulating CCR2⁺ IM may be important in supporting mCRC progression and have prognostic implications for human patients.

**Human CRC liver metastasis have increased expression of CCL2 and are infiltrated by immunosuppressive TAM**

To further understand CCR2⁺ IM mobilization in mCRC patients we examined serum and liver metastasis levels of the chemokine CCL2, which is responsible for CCR2 mediated IM recruitment. Analysis of the serum found elevated protein levels of CCL2 in mCRC patients compared to healthy controls (n = 15) and mCRC patients (n = 44). (B) Left panel, confocal microscopic images of human mCRC liver tumor showing CCL2 co-localization with Cytokeratin 20 (CK20) expressing neoplastic cells. Right panel, graph compares CCL2 gene expression by quantitative real time PCR between matched adjacent normal and mCRC liver tumors (n = 9). (C) Left panel, representative immunohistochemistry of CD68+ TAM (brown) in a hepatic mCRC tumor. Right panel, graph represents TAM as a percentage of CD45+ cells by flow cytometry analysis in matched adjacent normal and mCRC liver tumor samples (n = 4). (D) Left panel, graph depicts representative flow cytometry histogram of negative control (Blue) and CFSE labeled autologous CD8⁺ lymphocytes co-cultured alone (Orange), with CD14⁺ peripheral blood inflammatory monocytes (Green) or CD14⁺ TAM isolated from mCRC liver tumors (Red). Right panel, graph represents T-cell division index as determined between CD14⁺ cells isolated from matched pairs of peripheral blood and mCRC tumors (n = 3). Comparison between groups using a two-tailed unmatched t-test or a 2-tailed matched t-test for comparison of paired specimens were used to calculate p-values and shown as mean ± SD. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
to healthy controls (Figure 2A). This corresponded with an increase in CCL2 gene expression in CRC hepatic metastasis compared to adjacent normal liver (Figure 2B). Confocal microscopy showed co-localization of CCL2 with CK20, suggesting neoplastic mCRC cells and not the surrounding stroma are the primary source of CCL2 within tumors (Figure 2B & Supplemental Fig S1). At the metastatic site, mCRC liver tumors are heavily infiltrated by CCR2⁺ TAM relative to normal liver tissue (Figure 2C). To assess the immunosuppressive capacity of TAM, we isolated mCRC patient CD14⁺ IM from the peripheral blood and TAM from liver metastasis at the time of hepatic resection, which demonstrated patient TAM, but not circulating IM, potently suppressed autologous T-cell proliferation ex-vivo (Figure 2D). Together, this data supports the role of the CCL2/CCR2 chemokine axis in recruiting immunosuppressive TAM to the metastatic TME in mCRC.

**Inflammatory monocyte mobilization from the bone marrow is impaired in CCR2⁻/⁻ mice with metastatic CRC**

Similar to human disease, murine CRC liver metastasis demonstrate an increase in CCL2 expression compared to normal liver (Supplemental Figure S2). Flow cytometry analysis shows that CCR2⁻/⁻ mice with hepatic CRC metastasis have an increase in bone marrow CCR2⁺ IM compared to tumor bearing wild type mice (Figure 3A & Supplemental Fig S3A). This corresponded with a reduction in circulating CCR2⁺ IM in the peripheral blood of tumor bearing CCR2⁻/⁻ mice compared to wild type controls (Figure 3B and Supplemental Fig S3B). At the metastatic site, MC38 liver tumors from wild type mice displayed an abundant TAM infiltrate, which was significantly reduced in CCR2⁻/⁻ animals (Figure 3C). These results suggest that inhibiting CCR2 mediated recruitment of IM from the bone marrow prevents TAM accumulation in mCRC.

**Prolonged survival and mCRC liver tumor reduction in CCR2⁻/⁻ mice requires CD8⁺ TIL**

Overall, CCR2⁻/⁻ mice demonstrated an increase in survival following establishment of MC38 metastatic tumors compared to wild type controls (Figure 3D). As we found TAM to be potently immunosuppressive in human mCRC tumors, we hypothesized that the therapeutic efficacy of CCR2 blockade may be mediated through an anti-tumor immune response. In MC38 metastatic liver tumors, CCR2⁻/⁻ mice had an increase in both CD8⁺ and CD4⁺ TIL compared to controls (Figure 3E). However, while CCR2⁻/⁻ mice had a significantly less metastatic liver tumor burden compared to vehicle treated controls, the therapeutic efficacy was lost when CD8⁺ effector lymphocytes were depleted, supporting our hypothesis that targeting TAM in mCRC improves anti-tumor immunity (Figure 3F). Collectively, this data provides evidence that disrupting the CCL2/CCR2 chemokine axis prevents TAM accumulation and may represent an attractive treatment modality in mCRC.

**Treatment with a small molecule ccr2i reduces TAM and increases effector TIL in metastatic MC38 liver tumors**

Using a small molecule CCR2i alone or in combination with FOLFOX chemotherapy reveals that CCR2 blockade is effective at reducing tumor mediated mobilization of IM from the bone marrow (Figure 4A). While FOLFOX was insufficient to reduce TAM at the metastatic site, treatment with CCR2i, both alone and in combination with chemotherapy, effectively decreased the TAM infiltrate in MC38 mCRC liver tumors (Figure 4B). Similarly, we found no evidence of increased TIL in established MC38 liver metastasis with FOLFOX administration alone, while CCR2 blockade resulted in a concomitant increase in TIL and decrease in regulatory T-cells (CD4⁺, FoxP3⁺, CD25⁺) (Figure 4C). Furthermore, CCR2 inhibition resulted in an increase in CD69 surface expression on CD8⁺ TIL from MC38 liver metastasis, suggesting an activated phenotype, which was enhanced when combined with FOLFOX. Taken together, this suggests that CCR2 blockade of TAM enhances anti-tumor immunity by augmenting adaptive T-cell responses following chemotherapy.

**Ccr2i increases the efficacy of FOLFOX chemotherapy and improves survival in mice with mCRC**

Pharmacologic CCR2 blockade reduced tumor growth in both the MC38 and CT26 murine colorectal adenocarcinoma cancer cell lines compared to vehicle treated animals (Supplemental Fig S4). Analysis of viable tumor cells by bioluminescent imaging (BLI) and gross tumor weight found that CCR2i decreased established MC38 liver metastasis compared to vehicle treatment and this impact was further enhanced when CCR2i was administered in combination with FOLFOX chemotherapy (Figure 5A). Live in vivo assessment by BLI showed a significant reduction in malignant progression in mice with mCRC liver metastasis treated with CCR2i (Figure 5B). Likewise, gross metastatic MC38 liver tumor weights were significantly less in mice treated with CCR2i (Figure 5C). While treatment with FOLFOX alone was effective in reducing metastatic tumor burden, this effect was further enhanced when animals received chemotherapy in combination with CCR2 blockade. Following establishment of MC38 liver metastasis, neither FOLFOX (P = 0.13) nor CCR2i (p = 0.06) treatment alone increased survival compared to vehicle (Figure 5D). However, combined FOLFOX plus CCR2i treatment improved survival compared to vehicle treated animals (p < 0.001) and also demonstrated a survival advantage compared to CCR2i (p = 0.016) and FOLFOX (p = 0.037). In summary, these results collectively support targeting the CCL2/CCR2 chemokine axis in mCRC.

**Discussion**

Here we report several key observations that implicate the role of the CCL2/CCR2 chemokine axis in facilitating recruitment of immunosuppressive TAM to sites of liver metastasis in CRC patients, which support neoplastic growth and progression. First, increased pre-operative monocyte prevalence in the peripheral blood of mCRC patients is associated with worse clinical outcomes. Second, the majority of these
Peripheral monocytes are CCR2+ IM that are recruited to the tumor through the CCL2/CCR2 chemokine axis. Third, the metastatic site is heavily infiltrated by TAM, which display potent immunosuppressive properties. These findings provide a strong rationale for targeting CCR2 and in a mCRC model we demonstrate a reduction in TAM and metastatic tumor burden in both CCR2−/− mice following treatment with a small molecule CCR2i. Moreover, combination FOLFOX plus CCR2i therapy demonstrated increased anti-tumor activity and prolonged survival compared to either treatment alone, suggesting CCR2 blockade may augment conventional chemotherapy. Collectively, this data suggests that targeting bone marrow derived TAM recruitment represents a promising treatment modality in mCRC.

There is increasing evidence that tumors co-opt the CCL2/CCR2 pathway. Similar to our findings in mCRC, elevated preoperative monocytes correlates with a poor prognosis in several human cancers. Furthermore, neoplastic expression of CCL2 correlates with increased TAM infiltrate and worse prognosis in multiple solid tumors. Likewise, a
In this report we determined the effect of inhibiting CCR2 mediated recruitment of TAM in murine models of metastatic disease. In agreement with our previous work, targeting CCR2 decreased TAM and reduced malignant progression, however the authors report no evidence of an anti-tumor immune response following CCR2 inhibition. Several reasons may explain the difference in our findings. First, in the studies by Zhao, et al. the sample size was small and only CD3+ cells were assessed as compared to our data showing increased CD4+ and CD8+ TIL as well as subset analysis by surface marker expression. Additionally, the authors used a MC38 mCRC model in which a total splenectomy was performed, which may impact the adaptive immune response, compared to our model which allowed for the preservation of functional splenic tissue.

This evidence has supported clinical translation of strategies to reduce TAM in human cancer patients. Our group has previously reported the results from a clinical study in borderline and locally advanced pancreatic cancer using a small molecule CCR2i in combination with FOLFIRINOX, which showed that a small molecule CCR2i in combination with FOLFIRINOX reduced TAM and was associated with a promising treatment response compared to chemotherapy alone. Further clinical studies in metastatic pancreatic cancer using CCR2i combined with FOLFIRINOX (NCT02345408) are currently ongoing. Similarly, success targeting TAM with macrophage colony stimulating factor receptor (CSF1R) blockade has demonstrated efficacy in treating primary tumors, thus providing direct evidence to support our hypothesis that TAM have tumor promoting capacity in mCRC.

A: CCR2+ Inflammatory Monocytes

B: CCR2+ Tumor Associated Macrophages

C: Tumor Infiltrating Lymphocytes

**Figure 4.** Treatment with a small molecule CCR2i alone and in combination with FOLFOX chemotherapy changes the mCRC tumor microenvironment. (A) Graphs representing CCR2+ inflammatory monocytes in the bone marrow (Left) and peripheral blood (Middle) as a percentage of CD45+ cells in animals with MC38 liver metastasis following treatment with a small molecule CCR2i alone and in combination with FOLFOX chemotherapy. Graph illustrates changes in the blood to bone marrow inflammatory monocyte ratio (Right) in tumor bearing animals following treatment. (B) Graph represents mCRC liver tumor TAM as a percentage of total cells as determined by flow cytometry following treatment with a small molecule CCR2i alone and in combination with FOLFOX chemotherapy compared to vehicle. (C) Graphs depict CD8+ (Left) and CD4+ (Right) tumor infiltrating lymphocytes as percentage of total cells determined by flow cytometry from metastatic MC38 liver tumors. Comparison between multiple groups using ANOVA with Tukey post-test used to calculate p-values and shown as mean ± SD. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
**Figure 5.** CCR2i plus FOLFOX chemotherapy reduces established mCRC tumor burden and increases survival (A:) Representative BLI of in vivo and ex vivo livers from mice with established MC38 mCRC liver tumors. (B:) Graph represents region of intensity (ROI) as determined by BLI in mice with established MC38 hepatic metastasis following 15 days of treatment (n = 8–10 per group). (C:) Graph represents gross tumor weights from mice with established MC38 hepatic metastasis following 15 days of treatment (n = 10 per group). (D:) Overall survival following initiation of treatment in mice with established MC38 mCRC liver tumors (n = 9 mice per group). Comparison between multiple groups using ANOVA with Tukey post-test used to calculate p-values and shown as mean ± SD. Log rank test used for survival analysis. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
also shown promising results in synovial cell carcinoma clinical trials.\textsuperscript{39,40}

In summary, this study provides evidence that the CCL2/CCR2 chemokine pathway is utilized by mCRC to recruit TAM, which potently suppress anti-tumor immunity. CCR2 blockade alone reduces TAM and further improves survival when combined with FOLFOX chemotherapy in preclinical mCRC models. Data from clinical studies supports CCR2 blockade as a potential therapeutic adjunct in human patients and our results suggest that such strategies should be pursued in mCRC. Overall, this work provides compelling rationale for targeting CCR2 in the treatment of mCRC and offers additional insight into the role of TAM promoting metastatic disease.

**Material and methods**

**Preoperative monocyte analysis**

All patients (n = 221) with hepatic mCRC undergoing liver resection at Barnes-Jewish Hospital (St. Louis, MO) between 2005 and 2013 were identified from an IRB-approved, prospectively maintained database. The optimal cutoff point for percentage monocytes obtained from preoperative complete blood counts (CBC) within 30 days of liver resection was determined to be 8.9% using the log rank statistic with an adjustment for bias. Preoperative monocyte percent was used as a binary variable to compare overall survival following mCRC liver resection between high (≥8.9%) and low (<8.9%) cohorts. Univariate and multivariate analysis was performed to compare demographic and clinical characteristics.

**Patient blood, serum, and tumor collection**

Informed consent was obtained from all patients in accordance with institutional Human Studies Committee Protocol at Washington University School of Medicine (St. Louis, MO). Blood and serum was collected from healthy volunteers and patients with colorectal cancer liver metastasis prior to surgery. Peripheral blood was collected in heparinized tubes (BD BioScience) and mononuclear cells and serum isolated by Ficoll density gradient centrifugation. Human metastatic colorectal tumor and uninvolved liver tissue samples were snap-frozen in liquid nitrogen or mechanically and enzymatically dissociated, washed in phosphate buffered saline (PBS), and filtered over a 70 μM filter to create single cell suspensions. Isolated cells were assessed for viability using 0.4% Trypan Blue Solution (Fisher Scientific) and manually counted.

**T-cell suppression assay**

Peripheral blood mononuclear cells (PBMC) and single cell suspensions of mCRC liver tumor from matched patients were used to extract CD14\textsuperscript{+} cells using the EasySep CD14 Positive Selection Kit per the manufacturer’s instructions (STEMCELL Technologies). CD3\textsuperscript{+} T-cells were isolated from the CD14 depleted PBMC using the EasySep CD3 Positive Selection Kit (STEMCELL Technologies) and labeled with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies). CD14\textsuperscript{+} and CD3\textsuperscript{+} cells were plated in 96-well plates coated with human anti-CD3 (BioLegend) and incubated in complete media supplemented with human IL-2 for 72 hours at 37°C. CFSE dilution of CD8\textsuperscript{+} T-cells was analyzed via flow cytometry and the average number of cell divisions determined to calculate the division index using FloJo software (Tree Star, Inc).

**Cell lines and constructs**

The MC38 murine colorectal adenocarcinoma cell line has been previously described and was a kind gift from Dr. Scott Gerber.\textsuperscript{41} Pre-made lentiviral particles expressing the firefly luciferase 3 gene under the EF1α promoter with a Green Fluorescent Protein (GFP)-blasticidin fusion marker under the RSV promoter to allow for positive selection was used to transduce MC38 cells per the manufacturer’s instructions (GenTarget, Inc). Cells were incubated with blasticidin to create a stable GFP expressing cell line which was confirmed by flow cytometry.

**Animal studies and orthotopic model of hepatic mCRC**

Sex and age matched C57BL/6J and CCR2\textsuperscript{-/-} (B6.129S4-Ccr2\textsuperscript{mart1f/c}) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free vivarium under an approved animal studies protocol at Washington University School of Medicine (St. Louis, MO). To establish hepatic metastasis, mice were anesthetized with isoflurane and the spleen exposed through a left flank incision. The spleen was divided into two hemic-spleens using surgical clips (Ethicon), leaving the vascular pedicles intact. With a 27-gauge needle, 1.5 x10\textsuperscript{5} viable luciferase-labelled MC38 cells suspended in 50μL of phosphate buffered saline (PBS) were injected into the superior hemic-spleen, following which the vascular pedicle draining the cancer-contaminated hemic-spleen was ligated with a surgical clip and excised, leaving a functional hemic-spleen free of tumor cells.\textsuperscript{42} Animals were randomized 48 hours following tumor cell injections prior to treatment initiation. The high affinity CCR2 chemokine receptor antagonist, BMS CCR2 22 (Tocris), was dosed at 100 mg/kg and injected subcutaneously twice daily.\textsuperscript{53} FOLFOX therapy, consisting of 5-fluorouracil (30 mg/kg), oxaliplatin (10 mg/kg), and leucovorin (90 mg/kg) was given weekly via intraperitoneal injection. For CD8\textsuperscript{+} T-cell depletion experiments animals were administered 250 μg of anti-mouse CD8α monoclonal antibody (Clone 2.43; BioXcell) intraperitoneally starting 48 hours following tumor implantation and repeated every four days.

**Assessment of orthotopic liver metastatic tumor burden and survival**

Orthotopic MC38 liver metastatic tumor burden was measured by \textit{in vivo} bioluminescence imaging (BLI) on an IVIS Lumina (PerkinElmer). Mice were injected intraperitoneally with 150 mg/kg of D-luciferin in PBS (Gold Biotechnology) and imaged (1 to 60 sec exposure, bin8, FOV12.5cm, f/stop1,
open filter) under anesthesia using isoflurane. Total photon flux (photons/sec) was measured from fixed regions of interest (ROIs) over the liver using Living Image 3.2 software (PerkinElmer). For survival studies, events were recorded when mice lost >15% of body weight or per survival event.

**Flow cytometry**

Single cell suspensions were incubated with Fc receptor blocking solution followed by fluorophore-conjugated antibodies (BioLegend). For intracellular staining, cells were permeabilized with 1X permeabilization buffer per the manufacturer’s instructions (eBioscience). Flow cytometry was performed on an LSR II (BD Biosciences) and analyzed using Flow Jo Version X (Tree Star, Inc). See Supplementary Materials for complete list of antibodies (Supplemental Table S3).

**Immunofluorescence and immunohistochemistry**

A HM 325 microtome (Thermo Scientific) was used to cut 5 micrometer sections from embedded specimens. Tissue was baked at 70°C for 30 minutes then dewaxed in xylene followed by rehydration. Antigen retrieval was performed with a decloaking chamber (Biocare Medical) using citrate buffer (10mM Sodium Citrate, 0.05% Tween 20), then nonspecific antibody binding was blocked with Serum-Free Protein Block (DAKO). Primary antibodies were diluted in Antibody Diluent (DAKO) and incubated overnight at 4°C then washed and stained with fluorescently conjugated secondary antibodies diluted 1:200 for 30 minutes at room temperature. For immunohistochemistry, endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ in PBS and stained with primary and biotinylated secondary antibodies diluted 1:200. Antigen staining with VECTASTAIN Standard ABC Kit (Vector Laboratories) and Liquid DAB+ Substrate Chromagen System (DAKO) was performed and tissue sections were counterstained with Gill’s Hematoxylin (Fisher Scientific). An Olympus BX43 microscope with a DP80 camera was used to obtain images (Olympus).

**Measurement of CCL2 by quantitative real time PCR and enzyme-linked immunosorbent assay**

Total RNA was extracted from snap frozen tissue samples homogenized in Trizol (Thermo Scientific) with a Tissue Lyser LT bead mill (Qiagen) and reverse transcribed into cDNA using the High Capacity RNA-cDNA kit (ThermoFisher Scientific). TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assays (ThermoFisher Scientific) were used for quantitative real time PCR (qRT-PCR) performed on a 7500 Fast Thermal Cycler (Applied Biosystems). Gene expression was normalized to the housekeeper genes GAPDH, HPRT, and β-actin and analyzed using the manufacturer’s software (Applied Biosystems). Serum protein concentrations of CCL2 from healthy controls and patients with CRC liver metastasis were determined using the Human CCL2 Quantikine ELISA kit according to the manufacturer’s instructions (R&D Systems).

**Statistical analysis**

SAS version 9.1 (SAS Institute, Inc.) and GraphPad Prism version 4 (GraphPad Software, Inc.) were used to conduct statistical analysis. A p-value <0.05 was considered to be statistically significant. Normally distributed data was assessed using a 2-sided Student’s t-test and a 2-sided Mann-Whitney test used for non-Gaussian distributed data. For matched sample comparison, a 2-sided paired t-test was used. A one-way analysis of variance (ANOVA) using the Tukey post-test was performed for multiple comparisons. Survival analysis was performed using the log-rank test. Univariate Cox proportional-hazard models were used to evaluate the relationship between overall survival and various clinicopathological factors. The relationship between pre-operative blood monocyte level and overall survival was examined via multivariate Cox proportional-hazard model adjusting for significant clinicopathological factors identified in the univariate analysis. Proportional hazard assumption was examined by Cumulative Martingale residual plots, log of negative log plots, as well as correlations between Schoenfeld residual and rank of survival time. Kaplan-Meier curves were used to display survival differences between groups.

**Abbreviations**

Metastatic colorectal cancer (mCRC), Tumor microenvironment (TME), Tumor associated macrophage (TAM), inflammatory monocyte (IM), Tumor infiltrating lymphocyte (TIL)

**Acknowledgments**

TMN, JGG, BAB, SPG, and RCF recognize the Siteman Cancer Center Frontier CA009621 training grant in surgical oncology. TMN, JGG, BAB and RZF performed experiments and conducted analysis. BAK assisted in conducting experiments and editing the manuscript. WGH, DGD, SPG, DCL, and RCF provided assistance with experimental design and editing. WGH, DCL, and RCF provided funding support. TMN write the manuscript with input from all authors.

**Funding**

TMN, JGG, RZF, and BAK recognize support from the NIH T32 CA009621 training grant in surgical oncology. TMN, JGG, RZF, DGD, WGH, SPG, and RCF recognize the Siteman Cancer Center Frontier Fund. BAB and DCL recognize grant support from the NIH 5RO1CA168863.

**ORCID**

Julie G Grossman http://orcid.org/0000-0001-9323-5228

**References**

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. CA Cancer J Clin. 2016; 66: 7–30.
2. Lintoiu-Ursut B, Tulin A, Constantinoiu S. Recurrence after hepatic resection in colorectal cancer liver metastasis -Review article. J Med Life. 2015; 8: Spec Issue 12–14.
3. Chan KM, Wu TH, Cheng CH, Lee WC, Chiang JM, Chen JS, Wang YJ. Prognostic significance of the number of tumors and aggressive surgical approach in colorectal cancer hepatic metastasis. World J Surg Oncol. 2014; 12: 155. doi: 10.1186/1477-7819-12-155.
4. Van CE, Noldinger B, Cervantes A, Group EGW. Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. Ann Oncol. 2010; 21(Suppl 5): v93–7. doi: 10.1093/annonc/mdq222.

5. Noldinger B, Sorbye H, Glimelius B, Poston GI, Schlag PM, Rougier P, Bechstein WO, Primrose JN, Walpole ET, Finch-Jones M, et al. Perioperative chemotherapy with FOLFIRINOX and surgery versus surgery alone for resectable liver metastases from colorectal cancer (EORTC Intergroup trial 40983): a randomised controlled trial. Lancet. 2008; 371: 1007–1016. doi: 10.1016/S0140-6736(08)60455-9.

6. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006; 313: 1960–1964. doi: 10.1126/science.1129139.

7. Mlecnik B, Bindea G, Kirilovsky A, Angell HK, Obenauf AC, Tosolini M, Church SE, Maby P, Vasaturo A, Angelova M, et al. The tumor microenvironment and Immunoscoring are critical determinants of dissemination to distant metastasis. Sci Transl Med. 2016; 8: 327ra26. doi: 10.1126/scitranslmed.aad6352.

8. Le DT, Uram JN, Wang H, Barlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N Engl J Med. 2015; 372: 2509–2520. doi: 10.1056/NEJMoa1505096.

9. Stromnes IM, Greenberg PD, Hingorani SR. Molecular pathways: myeloid complicity in cancer. Clinical Cancer Research: an Official Journal of the American Association for Cancer Research. 2014; 20: 5157–5170. doi: 10.1158/1078-0432.CCR-13-0866.

10. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002; 420: 860–867. doi: 10.1038/nature01322.

11. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Reviews Immunol. 2012; 12: 253–268. doi: 10.1038/nri3175.

12. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144: 646–674. doi: 10.1016/j.cell.2011.02.013.

13. Pollard JW. Tumour-educated macrophages promote tumor progression and metastasis. Nature Reviews Cancer. 2004; 4: 71–78. doi: 10.1038/nrc1256.

14. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006; 124: 263–266. doi: 10.1016/j.cell.2006.01.007.

15. Sanford DE, Belt BA, Panni RZ, Mayer A, Deshpande AD, Skora AD, Laheru D, et al. Targeting tumour-associated macrophages with CCR2 inhibition in combination with FOLFIRINOX in patients with borderline resectable and locally advanced pancreatic cancer: a single-centre, open-label, dose-finding, non-randomised, phase 1b trial. Lancet Oncol. 2016; 17: 1007–1016. doi: 10.1016/S1470-2045(16)00078-4.

16. Li X, Yao W, Yuan Y, Chen P, Li B, Li J, Chu R, Song H, Xie D, Jiang X, et al. Targeting of tumour-infiltrating macrophages via CCL2/CCR2 signalling as a therapeutic strategy against hepatocellular carcinoma. Gut. 2017; 66: 157–167. doi: 10.1136/gutjnl-2015-310514.

17. Movahedi K, Laoui D, Gysemans C, Baeten M, Stange G, Van Den Bossche J, Mack M, Pipeleers D, In’t Veld P, De Baetselier P, et al. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. Cancer Research. 2010; 70: 5728–5739. doi: 10.1158/0008-5472.CAN-09-4672.

18. Shi S, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Reviews Immunol. 2011; 11: 762–774. doi: 10.1038/nri3070.

19. Matsuo K, Hom MS, Moeini A, Machida H, Takeshima N, Roman LD, Sood AK. Significance of monocyte counts on tumor characteristics and survival outcome of women with endometrial cancer. Gynecol Oncol. 2015; 138: 332–338. doi: 10.1016/j.ygyno.2015.05.019.

20. Shen SL, Fu SJ, Huang XQ, Chen B, Kuang M, Li SQ, Hua YP, Liang LJ, Peng BG. Elevated preoperative peripheral blood monocyte count predicts poor prognosis for hepatocellular carcinoma after curative resection. BMC Cancer. 2014; 14: 744. doi: 10.1186/1471-2407-14-744.

21. Bailey C, Negus R, Morris A, Ziprin P, Goldin R, Allavena P, Peck D, Darzi A. Chemokine expression is associated with the accumulation of tumour-associated macrophages (TAMs) and progression in human colorectal cancer. Clin Exp Metastasis. 2007; 24: 121–130. doi: 10.1007/s10585-007-9060-3.

22. DeNardo DG, Brennen DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, Gallagher WM, Wadhwani N, Keil SD, Junaid SA, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. Cancer Discovery. 2011; 1: 54–67. doi: 10.1158/2155-215X CD-10-0028.

23. Mitchell JB, Brennen DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, Belaygorod L, Carpenter D, Collins L, Piwnica-Worms D, et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. Cancer Research. 2013; 73: 1128–1141. doi: 10.1158/0008-5472.CAN-12-2731.

24. Lee CH, Espinosa I, Vrijlandhoven S, Subramanian S, Montgomery KD, Zhu S, Marinelli RJ, Peterse JL, Poulin N, Nielsen TO, et al. Prognostic significance of macrophage infiltration in leiomyosarcomas. Clinical Cancer Research: an Official Journal of the American Association for Cancer Research. 2008; 14: 1423–1430. doi: 10.1158/1078-0432.CCR-07-1712.

25. Jensen TO, Schmidt H, Moller HJ, Hoyer M, Maniecki MB, Sjoegren P, Christensen II, Steiniche T. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. J Clinical Oncology: Official Journal Am Soc Clin Oncol. 2009; 27: 3330–3337. doi: 10.1200/JCO.2008.19.9919.

26. Hasita H, Komohara Y, Okabe H, Masuda T, Ohiishi K, Lei XF, Beppu T, Baba H, Takeya M. Significance of alternatively activated macrophages in patients with intraperitoneal cholangiocarcinoma. Cancer Sci. 2010; 101: 1913–1919. doi: 10.1111/j.1349-7006.2010.01614.x.

27. Cavnar MJ, Turcotte S, Katz SC, Kuk D, Gonen M, Shia J, Allen PJ, Balachandran VP, D’Angelica MJ, Kingham TP, et al. Tumor-Associated Macrophage Infiltration in Colorectal Cancer Liver Metastases is Associated With Better Outcome. Ann Surg Oncol. 2017; 10.1245/s10434-017-5812-8.
Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Reviews Immunol. 2005; 5: 641–654. doi: 10.1038/nri1668.

Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. J Immunology. 2010; 184: 3106–3116. doi: 10.4049/jimmunol.0902661.

Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity. 2014; 41: 49–61. doi: 10.1016/j.immuni.2014.06.010.

Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol. 2017; doi: 10.1038/nrclinonc.2016.217.

Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. Oncotarget. 2016; 7: 28697–28710. doi: 10.18632/oncotarget.v7i19.

Chen X, Wang Y, Nelson D, Tian S, Mulvey E, Patel B, Conti I, Jaen J, Rollins BJ. CCL2/CCR2 Regulates the Tumor Microenvironment in HER-2/neu-Driven Mammary Carcinomas in Mice. PloS One. 2016; 11: e0165595. doi: 10.1371/journal.pone.0165595.

Cassier PA, Italiano A, Gomez-Roca CA, Le TourneauC, Toulmonde M, Cannarile MA, Ries C, Brillouet A, Müller C, Jegg AM, et al. CSF1R inhibition with emactuzumab in locally advanced diffuse-type tenosynovial giant cell tumours of the soft tissue: a dose-escalation and dose-expansion phase I study. Lancet Oncol. 2015; 16: 949–956. doi: 10.1016/S1470-2045(15)00132-1.

Tap WD, Wainberg ZA, Anthony SP, Ibrahim PN, Zhang C, Healey JH, Chmielowksi B, Staddon AP, Cohn AL, Shapiro GI, et al. Structure-Guided Blockade of CSF1R Kinase in Tenosynovial Giant-Cell Tumor. N Engl J Med. 2015; 373: 428–437. doi: 10.1056/NEJMoa1411366.

Corbett TH, Griswold DP Jr., Roberts BJ, Peckham JC, Schabel FM Jr. Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Research. 1975; 35: 2434–2439.

Soares KC, Foley K, Olino K, Leubner A, Mayo SC, Jain A, Jaffee E, Schulick RD, Yoshimura K, Edil B, et al. A preclinical murine model of hepatic metastases. Journal Visualized Experiments: JoVE. 2014; 51677.

Cherney RJ, Mo R, Meyer DT, Nelson DJ, Lo YC, Yang G, Scherle PA, Mandlekar S, Wasserman ZR, Jezak H, et al. Discovery of disubstituted cyclohexanes as a new class of CC chemokine receptor 2 antagonists. J Med Chem. 2008; 51: 721–724. doi: 10.1021/jm701488f.