Monocytic CCR2+ Myeloid-Derived Suppressor Cells Promote Immune Escape by Limiting Activated CD8 T-cell Infiltration into the Tumor Microenvironment

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Abstract

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells that accumulate during tumor formation, facilitate immune escape, and enable tumor progression. MDSCs are important contributors to the development of an immunosuppressive tumor microenvironment that blocks the action of cytotoxic antitumor T effector cells. Heterogeneity in these cells poses a significant barrier to studying the in vivo contributions of individual MDSC subtypes. Herein, we show that granulocyte-macrophage colony stimulating factor, a cytokine critical for the numeric and functional development of MDSC populations, promotes expansion of a monocytic-derived MDSC population characterized by expression of CD11b and the chemokine receptor CCR2. Using a toxin-mediated ablation strategy to target CCR2-expressing cells, we show that these monocytic MDSCs regulate entry of activated CD8 T cells into the tumor site, thereby limiting the efficacy of immunotherapy. Our results argue that therapeutic targeting of monocytic MDSCs would enhance outcomes in immunotherapy.

Introduction

A requirement for immunotherapy approaches against cancer is the expansion and entry of tumor antigen-specific T cells into the tumor microenvironment. However, established tumors develop an anti-inflammatory stroma and recruit immunosuppressive cells to the tumor site, both of which prevent immune eradication, even when tumor antigen-specific T cells are detectable in the periphery (1, 2). Myeloid-derived suppressor cells (MDSC; ref. 3), a heterogeneous population of myeloid cells, represent an immunosuppressive cell population that has been noted in many preclinical tumor models and in humans with cancer.

More than 20 factors have been described in MDSC expansion in the context of both murine and human tumors (4). Among these, granulocyte-macrophage colony stimulating factor (GM-CSF), an immune stimulatory cytokine in some situations, seems to also be critical for MDSC expansion and function. Recent negative results of randomized clinical trials using GM-CSF as an adjuvant underscore the importance of further characterizing the role of GM-CSF expanded MDSCs in immune-based therapies (5–7).

MDSCs consist of mononuclear, polymorphonuclear (PMN), and immature myeloid cells, with differential ability to suppress T-cell function via the coordinated function of iNOS and arginase (8). MDSC heterogeneity complicates the study of this population because of differing interpretations on how MDSC constituents fit within myeloid cell developmental lineages and contribute to tumor-induced immune suppression (9, 10). In mice, MDSCs express the myeloid antigens CD11b and Gr-1 (which stains both Ly6C and Ly6G) found on monotypic and neutrophilic cells. Other markers for these cells include F4/80, CD80, CD115, and IL4-R. More recently, Gr-1 or Ly6G with Ly6C expression have been found to identify a PMN population (CD11b+, Ly6G+/Ly6C high or Gr-1 high) and a mononuclear population (CD11b+, Ly6G+/Ly6C high or Gr-1 high) that expand to varying degrees and have differing suppressive capability in different tumor models, likely due to variations in tumor-secreted factors that mediate their expansion and/or activation. How these different MDSC subsets function in the context of distinct tumor-secreted factors remains poorly understood. Furthermore, tools to specifically track and deplete a single subset of MDSCs in vivo have not been developed.
In this study, we find that GM-CSF is critical for the expansion and function of monocyctic, but not granulocytic, MDSCs. We define monocyctic MDSCs by their coexpression of CD11b and CCR2. CCR2+ MDSCs are present in multiple tumor models and in patients with melanoma. Importantly, depletion of this cellular subset using recently described transgenic mice that enable the tracking and inducible depletion of CCR2+ cells defines a novel role for these cells in limiting antigen-specific T-cell entry into the tumor and thereby promoting tumor progression.

Methods

Mice

Mice were maintained and bred in a pathogen-free vivarium, and all procedures were carried out in accordance with institutional guidelines at Memorial-Sloan Kettering Cancer Center (MSKCC) under an IUCAC-approved protocol. C57/B6/J mice (females, 6- to 8-week-old) and C57BL/6 mice were obtained from The Jackson Laboratory. CCR2 knockout mice were obtained from Eric Pamer. CCR2-GFP reporter and CCR2-DTR-CFP depletor mice were generated by Tobias Hohl, as previously described in Hohl colleagues (17). Thy1.1+ pmel-1 TCR transgenic mice have been previously reported (18).

Cell lines and tumor challenge

B16F10 (referred to as B16), a mouse melanoma cell line of C57BL/6J origin, was obtained from the American Type Culture Collection (ATCC). B16, B16-GM, and TRAMP-C2 cells were cultured as described previously (19). EL4, EG7, and TRAMP-C2 cells were obtained from the American Type Culture Collection (ATCC). B16, B16-GM, and TRAMP-C2 cell lines were cultured as described previously (20, 21). EL4 and EG7 were cultured in RPMI supplemented with 7.5% heat-inactivated fetal calf serum (FCS; ATCC), 200 U/mL penicillin, and 50 mg/mL streptomycin (Invitrogen). For tumor challenge experiments, 1.25 × 106 B16 or B16-GM cells, 5 × 105 EL4 or EG7 cells, and 1 × 106 TRAMP-C2 cells were injected intradermally into the shaved flank of a mouse. Tumor growth was monitored every 2 to 3 days. Mice were sacrificed if tumors exceeded 1 cm in diameter or became ulcerated, or if mice showed signs of discomfort. For ex vivo analysis of immune infiltration into tumors or purification of tumor-infiltrating MDSC subsets mice were injected subcutaneously with the indicated numbers of tumor cells reconstituted in growth factor–reduced Matrigel (BD Biosciences) collagen matrix.

Antibodies and flow cytometry

Mouse tumor samples were finely chopped and treated with collagenase 2 mg/mL (Roche) and DNase 1 mg/mL (Sigma) in PBS at 37°C for 45 minutes. All tissues were washed through an 8-μm filter to generate single-cell suspensions in RPMI supplemented with 7.5% FCS. After BRC lysis (ACK lysis buffer; Lonza) when required, samples were washed in FACS buffer (PBS/1% BSA/2 mMOL EDTA), incubated in Fc block (CD16/32, clone 2.4G; BD Biosciences) and stained for 25 minutes at 4°C with the relevant Abs. Abs used for analysis of tumor infiltrate were Ly6C fluorescein isothiocyanate (FITC), CD4 PerCP, CD8 PE-Cy7, CD11b APC-Cy7 from BD Biosciences and FoxP3 APC (intracellular) from eBioscience. Additional Abs used for T-cell adoptive transfer experiments were Ly6G PE, Thy1.1 PerCP, CD11b PerCp-Cy5.5, CD45 APC-Cy7 from BD Biosciences and Ly6C AF647 from AbD Serotec. Live/Dead fixable aqua-dead stain or 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) was added to cells, as appropriate, to assess viability prior to acquisition on an LSRS flow cytometer. For human samples 5 × 10⁶ peripheral blood mononuclear cells (PBMC) from melanoma patients or healthy donors were washed with 2 mL FACS buffer (PBS containing 2% bovine serum albumin and 0.05 μmol/L EDTA). The following antibodies were then added for 20 minutes at 4°C: Lineage (CD3/CD16/CD19/CD20/CD56) cocktail FITC (special-ordered BD Pharmingen), CD14-PerCP Cy5.5, CD11b-APC Cy7, CD33-PE-Cy7 (BD Pharmingen), HLA-DR-ECD (Beckman Coulter), IL-4Rα-PE (R&D Systems), and CD192 (CCR2)-Alexa Fluor 647 (Biolegend). Isotype controls included the appropriate fluorochrome-conjugated mouse IgG1, IgG1, IgG2a, or IgG2b,k (BD Pharmingen, Beckman Coulter, R&D Systems, or Biolegend). The stained cells were detected using a CyAn flow cytometer. All analyses were carried out using FlowJo software (Treestar).

Purification of MDSCs

Mouse tumor–infiltrating or lymphoid tissue CD11b+ cells were obtained from single-cell suspensions generated as in the previous section. Tumor cells were subsequently separated from debris over a Percoll (Sigma Aldrich) gradient. B cells were depleted from splenocytes using CD19 microbeads and LD columns according to the manufacturer’s protocol (Miltenyi Biotec) to enrich myeloid fractions. Cells were stained with CD11b PerCP-Cy5.5, CD45 APC-Cy7, and DAPI for flow sorting on a Cytomation MoFlo or BD FACS-Diva Cell Sorters. Purity of FACS sorted populations was 90 to 95%.

For human samples thawed PBMCs were resuspended in 0.5% human serum PBS at 5 × 10⁶ cells/mL and incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) at a final concentration of 0.5 μmol/L for 5 minutes at room temperature. Cells were washed 3 times in 0.5% Human serum PBS. A total of 3 × 10⁶ PBMCs were stained with 10 μL of anti-human CCR2-PE antibody for 15 minutes at 4°C and washed in 0.5% PBS PBS twice. The cells were then depleted of CCR2-PE+ cells with anti-PE MicroBeads (Miltenyi Biotec) according to the instructions supplied by the manufacturer. These were used as effectors. CCR2+ cells were also flushed from the columns for use as suppressors where indicated. Human PBMCs were collected using a Memorial Hospital Institutional Review Board–approved biospecimen protocol (MH # 00-144) for study of correlative immunologic markers in patients with melanoma.

T-cell preparations

Spleen and lymph nodes were passed through an 8-μm filter to generate a single-cell suspension. These were subsequently subjected to BRC lysis and magnetically purified using anti-CD8 (Ly-2) microbeads according to the manufacturer’s...
protocol (Miltenyi Biotec). Purity was assessed by flow cytometry and greater than 90%. Cells were labeled with 1 or 10 mmol/L CFSE; (Invitrogen) for in vitro or in vivo studies, respectively. Activated CD8 T cells were generated by culturing splenocytes with soluble α-CD3, 1 μg/mL (145–2C11) and α-CD28, 2 μg/mL (37N) for 72 hours. Recombinant human interleukin (IL)-2, 30 units/mL (Chiron) was added for the final 24 hours of culture. CD8+ cells were subsequently positively selected with anti-CD8 microbeads prior to injection via tail vein.

Suppression assay
A total of 1 × 10⁷ CFSE-labeled splenocytes or CD8+T cells were plated in complete media (RPMI 1640, 10% FCS, 200 units/mL penicillin, 50 mg/mL streptomycin) supplemented with 2 mmol/L L-glutamine and 0.05 mmol/L β-mercaptoethanol onto round bottom 96-well plates coated with 1 μg/mL α-CD3 and 5 μg/mL α-CD28. MDSCs were added in 1:1 ratios unless otherwise indicated. To assess contact dependence, MDSCs were placed into transwell inserts with 0.4 μmol/L polycarbonate membranes (Corning HTS Transwell; Corning).

For human cells, 2 × 10⁵ CFSE-labeled CCR2−/−PBMCs with or without CCR2+ cells were cultured in 96-well flat bottom α-CD3–specific Ab-coated plates (OKT3, 100 mcL at 0.5 μg/mL for 2 hours at 37°C) in RPMI 1640 medium supplemented with 10% FBS and IL-2 (10 IU/mL; Roche). After 5 days, cells were harvested, stained with CD3-PECy7, CD4-ECD, and CD8-APCCy7 (BD Pharmingen), and CFSE signal of gated CD8+ T cells (CD3+ CD4−) was measured by flow cytometry.

Cytokine measurements
Mouse blood was obtained by retro-orbital puncture when tumors were 0.5 to 1 cm in diameter. Blood was allowed to clot for 30 minutes on ice and centrifuged at 3,600 RPM for 5 minutes and serum was aspirated. Tumors were weighed, placed in 500 ml PBS, and treated in a bead homogenizer for 5 minutes. Debris was removed by 2 rounds of centrifugation at 14,000 RPM to obtain cytokines in solution. Cytokine levels were measured using FlowCytomix beads (eBioscience) according to the manufacturer's instructions. Samples were acquired on an LSRII flow cytometer and analyzed using FlowCytomixPro software.

Statistics
All values shown in graphs represent the mean ± SEM. Statistical differences between different groups were determined by a 2-tailed Student's t test. P < 0.05 was considered significant.
statistically significant. Correction for multiple comparisons was not carried out due to the hypothesis generating nature of these studies. Differences between tumor growth curves were assessed using ANOVA analysis. All graphs and statistical calculations were done using Prism software (GraphPad Software).

Results

Chronic GM-CSF promotes tumor growth

In prior studies, we observed that live B16 murine melanoma cells engineered to secrete GM-CSF promote concomitant immunity in vivo (1, 22, 23). In this model, a primary tumor that secretes GM-CSF is capable of protecting against a second tumor challenge. This effect is CD8^+ T-cell dependent. However, we found it surprising that the primary GM-CSF secreting B16 tumors (B16-GM) continue to grow despite the presence of protective CD8^+ T-cell immunity. To study this further, we assessed the effects of GM-CSF on tumor growth kinetics by challenging C57BL/6 mice with B16 or B16-GM tumors. We observed that B16-GM led to a more rapid tumor growth in vivo (Fig. 1A), suggesting that the sum effect of chronic GM-CSF exposure is to promote tumor growth. We found both cell lines had similar growth kinetics in vitro, with B16 having a slightly more rapid doubling time than the GM-CSF secreting counterpart (Fig. 1B) making it unlikely that GM-CSF is exerting a direct proliferative effect on the tumor cells. Furthermore, B16 and B16-GM growth differences were abrogated in CSF2 receptor knockout mice, which lack the common beta subunit shared by GM-CSF, IL-3, and IL-5 (Fig. 1C). These data suggested that chronic GM-CSF exposure promotes tumor growth indirectly via effects on host cells. Interestingly, both tumor cell lines grew at a slightly increased rate in CSF2 knockout mice, suggesting a global role for this shared receptor in controlling tumor outgrowth (data not shown).

Tumor-derived GM-CSF recruits a cellular infiltrate dominated by myeloid-derived suppressor cells

Because indirect effects on host cells seemed to explain the observed tumor growth kinetics, we examined the effects of chronic GM-CSF exposure on tumor-infiltrating immune cell subsets in B16 and B16-GM tumors. B16-GM tumor growth was noted to increase the number of myeloid cells both at the tumor site and in the spleen (Fig. 2A and B), whereas an increase in the absolute number of regulatory T cells (Tregs) was only observed in the spleen (Fig. 2A). Significantly diminished CD8^+ T-cell infiltration was noted at the tumor site with...
mildly diminished numbers seen systemically (Fig. 2A). To test the prediction that the myeloid cells infiltrating the B16-GM tumor site were MDSCs, we carried out T-cell suppression assays comparing equivalent numbers of CD11b cells purified by flow cytometry from B16 or B16-GM tumors for CD8$^+$ T-cell suppressive capability. This showed that CD11b$^+$ cells isolated from the B16-GM tumor site were MDSCs (Fig. 2C). In contrast, CD11b$^+$ cells isolated from the spleens of B16-GM-bearing animals did not suppress CD8$^+$ T-cell proliferation, suggesting that MDSCs at the tumor site were a suppressive subset of CD11b$^+$ cells present in the tumor microenvironment. Importantly, CD11b$^+$ cells purified from the B16 tumor or from the spleen of B16 tumor-bearing mice were incapable of suppressing CD8$^+$ T-cell proliferation. In sum, these results showed that B16-GM recruits an immune-inhibitory cellular infiltrate characterized by a CD11b$^+$ MDSC population and a relative paucity of CD8$^+$ T cells.

**GM-CSF expands monocytic CCR2$^+$ MDSCs**

To investigate whether GM-CSF expanded MDSCs are granulocytic or monocytic cells, we focused on CCR2 expression, as this chemokine is required for monocyte trafficking from the bone marrow and is not expressed on granulocytes (24). Furthermore, monocytic MDSCs have been shown to depend on CCR2-mediated signals for tumor trafficking (25). CCR2-specific antibodies have been reported but are not commercially available (26). Using a CCR2-GFP reporter mouse strain, we found that both CCR2$^+$CD11b$^+$ and CCR2$^-$/CD11b$^+$ myeloid cells are systemically expanded by GM-CSF, with a preferential accumulation of CCR2$^+$CD11b$^+$ cells at the tumor site (Fig. 3A). As expected, sorting cells based on CCR2 expression

![Figure 3.](https://example.com/figure.jpg)

**Figure 3.** CCR2 reporter expression segregates a monocytic CD11b$^+$ MDSCs in the tumor-bearing host. CCR2 reporter mice (4–8 per group) were inoculated with B16-GM or B16 tumor cells in Matrigel. Tumors and spleens were harvested after 2 weeks or when tumors were of equivalent size (500–800 mg) and analyzed for GFP expression in CD45$^+$ CD11b$^+$ cells by flow cytometry. The quantity of CD11b$^+$GFP$^+$ (CCR2$^+$) and GFP$^-$ (CCR2$^-$) cells as a percentage of the immune infiltrate (%CD45$^+$ cells) is shown. One representative spleen sample is depicted to show each population. Data are composite of 2 separate experiments (A). In separate experiments cytospins of flow-sorted cells were stained with a Diff-Quick stain set (Dade Behring, inc.) and evaluated at 40× magnification (B). Single-cell suspensions harvested from tumors and spleens 2 weeks after tumor inoculation were flow sorted for CD11b$^+$CD45$^+$ cells and then additionally sorted for CCR2$^+$ or CCR2$^-$ populations based on GFP reporter expression. Each of the 3 subsets was individually tested for its ability to suppress the proliferation of CD8$^+$ T cells stimulated with α-CD3/α-CD28 antibody (Stim). Stim$^-$ are wells without CD11b$^+$ cells and Stim$^+$ are wells without MDSCs or antibody. Proliferation was measured after 72 hours of coculture with the indicated ratio of MDSCs (C). *P < 0.05.
CCR2 expression can be applied more broadly to characterize monocytic MDSCs.

**MDSC suppressive function is CCR2 independent**

The observations that CCR2 reporter expression defines an MDSC subset led us to evaluate the role of this receptor in MDSC function. CCR2 is critically important for monocyte egress from the bone marrow in the setting of acute inflammatory events, such as infection, and a role for this molecule in myeloid cell trafficking to tumors has also been proposed (24, 25). Therefore, we crossed the CCR2 reporter mice (in which the CCR2 locus is disrupted by the GFP gene) onto a CCR2-/- background to identify MDSCs that lacked functional CCR2.

**Figure 4.** GM-CSF expands and activates monocytic CCR2<sup>+</sup> CD11b<sup>+</sup> MDSCs that suppress CD8<sup>+</sup> T-cell proliferation via the coordinated action of iNOS and arginase in a contact-dependent fashion. CCR2-GFP mice (3–6 per group) were subcutaneously inoculated with B16-GM embedded in Matrigel. Single-cell suspensions harvested from spleens 2 weeks later were flow sorted for CD11b<sup>+</sup>CD45<sup>-</sup> CCR2 reporter + cells (MDSCs), which were added to CD8<sup>+</sup> T cells stimulated with α-CD3/α-CD28 antibody. Proliferation was measured after 72 hours of coculture in the indicated conditions (A). CD8<sup>+</sup> T-cell suppressive capability measured with MDSCs in contact with CD8<sup>+</sup> T cells or placed inside a transwell (0.4 μm insert) (A). CD8<sup>+</sup> T-cell proliferation in the presence of inhibitors of iNOS (L-NMMA) and/or arginase (nor-Noha); proliferation is normalized across assays to the baseline proliferation in the absence of MDSCs (B). One representative experiment of 2 similar replicates is shown in A. In B, combined results from 3 individual assays are shown. *P < 0.05.
We found a 50% reduction in the number of GFP⁺ MDSCs at the tumor site with similar reductions seen in the blood and spleen (Fig. 5A and B). A reciprocal increase of GFP⁺ MDSCs in the bone marrow was seen. These findings are consistent with results from models of infection and suggest against additional CCR2 dependence for MDSC trafficking from the vasculature to the tumor site. Suppression assays with CCR2 reporter⁺ MDSCs purified from B16-GM tumor-bearing animals from both wt and CCR2−/− mice revealed equivalent suppressive capability (Fig. 5C), showing that on a per cell basis, signaling through CCR2 was not required for MDSC activity.

**CCR2⁺ cells inhibit the therapeutic efficacy of adoptive T-cell therapy**

To evaluate the effects of CCR2⁺ MDSCs *in vivo*, we utilized a mouse strain engineered with a transgene that expresses the diphtheria toxin receptor under control of the CCR2 promoter (CCR2 depleter mice). In CCR2 depleter mice, diphtheria toxoid (DT) administration results in more than 99% depletion of bone marrow and tissue monocytes within 12 hours of injection (17). To segregate any potential heterogeneity in CCR2 expression on nonmyeloid cells, we utilized adoptive transfer of melanoma–specific CD8 T cells, which lack DTR transgene expression and have functional CCR2 for these experiments. Splenocytes from pmel-1 T-cell receptor transgenic mice were treated *in vitro* with α-CD3/α-CD28 and IL-2 to obtain a uniformly activated CD8⁺ T-cell population specific for the melanosomal antigen gp100. After 3 days, CD8⁺ T cells were magnetically sorted from these cultures and transferred into B16-GM tumor–bearing mice that had been reconstituted with CCR2-DTR Tg positive or negative bone marrow. All mice were treated with DT as indicated in the experimental schema (Supplementary Fig. S3). DT treatment resulted in near complete elimination of CD11b⁺Ly6C⁺ (monocytic) cells and partial ablation of CD11b⁺Ly6Clo cells (macrophages) with preservation of CD11b⁺Ly6G⁺Ly6C intermediate (granulocytic) cells at the tumor site (Fig. 6A and Supplementary Fig. S3). Depletion of CCR2⁺ cells resulted in significantly greater accumulation of pmel T cells at the tumor site, with a trend toward diminished T cells seen in the draining lymph node (Fig. 6B and C). The increased antigen-specific T-cell accumulation in the tumor resulting from the depletion of CCR2⁺ cells at this site resulted in significant delay in tumor growth in a parallel experiment (Fig. 6D). Depletion of CCR2⁺ cells alone...
lead to a growth delay, but, importantly, the combined effect was greatest with the highest pmel cell dose, underscoring the active role of antigen-specific immunity in mediating an antitumor effect (Fig. 6E).

**Discussion**

There has been a great deal of interest in the immunostimulatory properties of GM-CSF in autoimmune diseases.
and for immunotherapy in cancer (29, 30). More than 15 years ago, Dranoff and colleagues showed that GM-CSF, in the context of γ-irradiated tumor cells, elicits potent immune responses in a murine model of melanoma (22). This prompted the study of GM-CSF as an adjuvant to whole tumor, DNA, and peptide vaccination with promising results in a number of animal tumor models (23, 31–34). Similar strategies were safely carried out in early phase clinical trials and immune responses were elicited (35–38). However, in more recent randomized clinical trials, GM-CSF was found to have detrimental effects on both immune responses and clinical outcomes (5–7), a finding that may be related to the expansion of MDSCs. Several groups have observed that GM-CSF dose and duration of exposure may mediate MDSC expansion (16, 28, 39–42). However, a direct connection between MDSC expansion and the failings of GM-CSF as a vaccine adjuvant has not been established.

To date, a specific characterization of GM-CSF expanded MDSCs or a direct examination of MDSC effects on tumor immunity in vivo has been hampered by a lack of reagents to specifically target this population. Using a transgenic mouse with a CCR2 promoter–driven GFP reporter, we show that chronic GM-CSF exposure expands and activates monocyte-derived MDSCs that express CCR2 and are direct descendents of CCR2-expressing cells. Monocytic CCR2-expressing MDSCs are present in multiple tumor types and in patients with melanoma (Fig. 3C, 7, and Supplementary Fig. S1), suggesting that CCR2 is a generally applicable marker of this cell population. Additional studies are warranted to define the precise contribution of GM-CSF to MDSC function in tumor-bearing hosts, as well as other pathologic conditions associated with MDSC accumulation.

In our depletion studies, we found that bone marrow–derived CCR2+ cells promote tumor growth and diminish the therapeutic effect of adoptively transferred activated antigen-specific CD8+ T cells by preventing their infiltration into the tumor site (Fig. 6). Our studies utilized adoptively transferred DTR transgene negative T cells, a clinically valid approach, to examine the effects of CCR2+ cell depletion (43). The use of adoptively transferred DTR transgene negative T cells excludes the possible effects of DT administration on endogenous CD8+ T cells and permits the conclusion that CD8+ T-cell accumulation at the tumor site is inhibited by CCR2+ MDSCs (26).
Although CCR2 enables the characterization of an MDSC subtype, it is also found on a subset of CD4 and CD8 T cells (26). In addition, in both naive mice and B16 tumor–bearing mice, neither CCR2 reporter expression nor an MDSC population defined by other markers (CD11b/Gr1, CD11b/F4/80, or CD11b/CD115) identifies a functional MDSC subtype (ref. 39; and data not shown), presumably because the signals required to activate the suppressive program are not present under these conditions. Furthermore, our experiments in CCR2−/− mice show that MDSCs retain functionality, suggesting that signaling through this receptor is not required for suppressive function (Fig. 5). Thus, CCR2 expression, but not signaling, on MDSCs could be used for targeting cell lineage–specific reagents aimed at manipulating MDSC function in vivo.

It has been shown that CCR2 is important for MDSC trafficking to the tumor site (25). We have also observed that accumulation of CCR2+ MDSCs at the tumor site is significantly diminished in CCR2−/− mice. Our numeric data suggests that this is because these cells are less efficient at exiting the bone marrow space. A role for CCR2 in MDSC trafficking from the blood and lymphoid organs into tumors is not supported by our findings. CCR2 is required for monocyte egress from the bone marrow in the setting of acute inflammation, but other pathways may also play a role in the more chronic setting of tumor growth explaining the residual MDSC accumulation observed in B16-GM tumors (24, 25). It has been shown that the spleen can serve as a reservoir for monocytes that can be released in stress conditions in response to angiotensin II (44). We did not observe further decreases in MDSC quantity in splenectomized CCR2−/− mice challenged with B16-GM tumors (data not shown), thus not supporting the notion that residual MDSCs at the tumor site in CCR2−/− mice are released from the spleen. Additional studies are needed to define the full complement of pathways used by CCR2+ MDSCs for trafficking to the tumor site.

In summary, we have found that CCR2 expression defines a monocyte-derived population of suppressive myeloid cells that regulates activated CD8+ T-cell entry into the tumor site. Our results suggest that combined targeting of CCR2+ MDSCs as part of an immune-based treatment strategy may improve the outcome of immunotherapy in the tumor-bearing host.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors’ Contributions

A.M. Lesokhin designed and carried out research, analyzed data, and wrote the article; T.M. Hohl contributed vital reagents, designed research, analyzed data, and contributed to writing the article; S. Kitano, C. Cortez, D. Hirschhorn-Cyerman, and G.A. Rizzuto carried out research and contributed to writing the article; F. Avogadri and J.J. Lazarus conducted experiments; E.G. Pamer contributed vital reagents and contributed to writing the article; A.N. Houghton designed experiments, analyzed data, and contributed to writing the article; T. Merghoub and J.D. Wolchok designed research, analyzed data, and wrote the article.

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