Tumor-Infiltrating Monocytic Myeloid-Derived Suppressor Cells Mediate CCR5-Dependent Recruitment of Regulatory T Cells Favoring Tumor Growth

Eva Schlecker, Ana Stojanovic, Christian Eisen, Christian Quack, Christine S. Falk, Viktor Umansky and Adelheid Cerwenka

*J Immunol* 2012; 189:5602-5611; Prepublished online 14 November 2012;
doi: 10.4049/jimmunol.1201018
http://www.jimmunol.org/content/189/12/5602
Tumor-Infiltrating Monocytic Myeloid-Derived Suppressor Cells Mediate CCR5-Dependent Recruitment of Regulatory T Cells Favoring Tumor Growth

Eva Schlecker,* Ana Stojanovic,* Christian Eisen,† Christian Quack,‡ Christine S. Falk,§ Viktor Umansky,§,* and Adelheid Cerwenka*

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of myeloid cells in cancer patients and tumor-bearing mice that potently inhibits T cell responses. During tumor progression, MDSCs accumulate in several organs, including the tumor tissue. So far, tumor-infiltrating MDSC subpopulations remain poorly explored. In this study, we performed global gene expression profiling of mouse tumor-infiltrating granulocytic and monocytic (MO-MDSC) subsets compared with MDSCs from peripheral blood. RMA-S lymphoma-infiltrating MO-MDSCs not only produced high levels of NO and arginase-1, but also greatly increased levels of chemokines comprising the CCR5 ligands CCL3, CCL4, and CCL5. MO-MDSCs isolated from B16 melanoma and from skin tumor–bearing ret transgenic mice also expressed high levels of CCL3, CCL4, and CCL5. Expression of CCR5 was preferentially detected on regulatory T cells (Tregs). Accordingly, tumor-infiltrating MO-MDSCs directly attracted high numbers of Tregs via CCR5 in vitro. Intratumoral injection of CCL4 or CCL5 increased tumor-infiltrating Tregs, and deficiency of CCR5 led to their profound decrease. Moreover, in CCR5-deficient mice, RMA-S and B16 tumor growth was delayed emphasizing the importance of CCR5 in the control of antitumor immune responses. Overall, our data demonstrate that chemokines secreted by tumor-infiltrating MO-MDSCs recruit high numbers of Tregs revealing a novel suppressive role of MDSCs with potential clinical implications for the development of cancer immunotherapies.

Multiple mechanisms of immune suppression accompanying tumor development have been reported (1). Alterations in myelopoiesis that occur during tumor growth lead to the accumulation of myeloid-derived suppressor cells (MDSCs). MDSCs represent a heterogeneous population of myeloid cells at different stages of differentiation (2, 3). In mice, MDSCs are characterized by the coexpression of the cell-surface markers Gr-1 and CD11b. Within this population, two MDSC subsets with distinct morphological features have been identified comprising monocytic MDSCs (MO-MDSCs) that express high levels of Ly6C and F4/80 and granulocytic MDSCs (PMN-MDSCs) expressing Ly6G and no F4/80 (4, 5). MDSCs potently inhibit T cell proliferation and cytokine production in vitro (3, 6, 7). Several mechanisms for their direct suppressive activity on T cells were reported, such as the production of reactive oxygen species (ROS) and nitrogen species and arginase-1 (8) or by depriving cysteine from the environment (9). So far, most studies investigated the accumulation, subset distribution, and regulatory role of MDSCs in bone marrow, blood, and spleen. Comparatively little is known about their function within the tumor tissue of solid malignancies including lymphoma and melanoma.

Regulatory T cells (Tregs) represent another suppressive population that potently inhibits T cell and NK cell function in cancer patients and tumor-bearing mice (10–12). CD4+CD25+ Tregs expressing the transcription factor Foxp3 inhibit autoimmune responses and promote tumor progression (13, 14). Accordingly, the depletion of Tregs results in improved antitumor immune responses and delays tumor growth in many tumor models (15). High numbers of Tregs in tumors such as lymphoma (16) are often associated with poor prognosis for cancer patients. Several mechanisms of Treg recruitment to tumors were reported. In breast and prostate cancer patients, CCL22 present in the tumor microenvironment mediated Treg trafficking in a CCR4-dependent manner (17, 18). Recently, it was shown that tumor-derived CCL28 mediates CCR10-dependent Treg recruitment in ovarian cancer (19). In a mouse model of pancreatic adenocarcinoma that produces CCL5, increased Treg numbers were associated with CCR5 expression by Tregs (20). However, whether additional cells, like tumor-infiltrating immune cells, mediate CCR5-dependent Treg recruitment remains elusive.

Recent evidence demonstrates that MDSCs and Tregs interact with each other to build a suppressive network during antitumor immune responses. In this context, it was shown that CD40 (21), CD80 (22), and arginase-1 expression (23) by MDSCs are involved in the induction of Treg development and T cell tolerance in spleen, bone marrow, and ascites. It has been recently reported that CCL5 gene expression in blood-derived MDSCs was downregulated by treatments with tolerating regimen affecting the
intragraft localization of Tregs (24). Whether tumor-infiltrating MDSCs interact with Tregs within the tumor tissue of solid tumors and influence each other’s accumulation and recruitment remains to be investigated.

In our study, we performed global gene arrays of tumor-infiltrating MDSC subsets to gain insight in their function within the tumor tissue. Our data demonstrate that tumor-infiltrating MO-MDSCs produce high levels of the CCR5 ligands, CCL3, CCL4, and CCL5 and directly attract Tregs in a CCR5-dependent manner. Accordingly, in CCR5-deficient mice, decreased Treg levels were detected in the tumor tissue that was associated with delayed tumor growth. Overall, our results reveal the recruitment of Tregs as a novel suppressive mechanism mediated by tumor-infiltrating MO-MDSCs.

Materials and Methods

Mice and tumor models

C57BL/6 (wild-type [wt]) mice were purchased from Charles River Laboratories (Sulzfeld, Germany, and Erembedogeum, Belgium). CCR5−/− mice (at least 10 backcross generations) and congenic C57BL/6-CD45.1−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. Mice (C57BL/6 background) that overexpress the human TNF receptor II transmembrane in the melanoma cell line under the control of the mouse metallothionein I promoter enhancer were kindly provided by Dr. I. Nakashima (Chubu University, Aichi, Japan) (25). Mice were housed in specific pathogen-free conditions and used in experiments at 6–8 wk of age. All experiments were performed according to local animal experimental ethics committee guidelines (Regierungspräsidium, Karlsruhe, Germany). The C57BL/6 syngeneic MHC class I-deficient lymphoma cell line RMA-S and the melanoma cell line B16BL6 (American Type Culture Collection, ATCC) were cultured in RPMI 1640 and DMEM, respectively, with 10% FCS, 1% l-glutamine, 1% penicillin, and 1% streptomycin (Invitrogen). RMA-S (5 × 106 or 106 as indicated) or B16 (5 × 105) cells were incubated into the right flank of mice. Tumors were measured every third day. A total of 105 or 106 as indicated) or B16 (5 × 105) cells were injected into the lower hind footpads of C57BL/6 mice expressing CD45.2 were sorted by FACS and placed in 600 μl primary cell medium in the lower wells of an HTS Transwell-24 (Corning) with a 5-μm pore size. After 18 h, pre-activated Tregs (2 × 105 in 200 μl) were added to the upper chamber and cultured for 5 h. Migrated Tregs were harvested from the lower chamber and stained with anti-CD45.1 mAbs. After staining, Accudrop Counting Beads (BD Biosciences) were added, and the numbers of cells were determined by CD45.1 expression using the FACSCalibur flow cytometer (BD Biosciences). Purified Tregs from CCR5−/− mice were labeled with CFSE, and numbers of migrated cells were distinguished by CFSE labeling after addition of Accudrop Counting Beads (BD Biosciences).

Morphological analysis

Cytospins of FACS-sorted MDSC subsets were prepared using poly-l-lysine-coated microscopic glass slides (Sigma-Aldrich). Cells were fixed in 4% paraformaldehyde in PBS for 10 min. The nuclei were stained with Mayer’s Hematoxylin (DakoCytoptation), and counterstaining of the cytosol was performed with 10% eosin Y (Applichem). Images were acquired using the Olympus BX51 microscope with ×200 magnification (Olympus).

Characterization of MDSC subsets

Arginase activity. A total of 6 × 105 FACS-sorted MDSCs were washed in PBS. Pellets were resuspended in 100 μl lysis buffer (0.4% Triton X-100 with 10 mM Tris-HCl and protease inhibitors) for 30 min at 4˚C. Arginase activity in the supernatants was measured using the QuantiChrom arginase assay kit (BioAssay Systems).

ROS production. Single-cell suspensions from blood and tumor were incubated at 37˚C in PBS containing 2.5 μM oxidative-sensitive dye DCFDA (Invitrogen) with or without 30 ng/ml PMA for 30 min. Subsequently, cells were stained with anti-Gr-1 and anti-F4/80 mAbs and analyzed by flow cytometry.

Bioplex protein array

A total of 5 × 105 FACS-sorted MDSCs were cultured in 100 μl primary cell medium for 24 h. Supernatants were collected and further diluted 1:1 with Bioplex Sample Diluent (Bio-Rad). Chemokine release was simultaneously determined with the Bioplex System (Bio-Rad) according to the manufacturer’s instructions. Standard curves and concentrations were calculated with Bioplex Manager 4.1.1 (Bio-Rad) on the basis of the five-parameter logistic plot regression formula.

Gene expression profiling and data analysis

MDSC subsets were sorted by flow cytometry from peripheral blood of PBS-injected naive mice (poled, n = 30) or from blood and tumor of RMA-S-injected mice (poled, n = 25) 19 d after tumor cell inoculation. The purity of sorted cells (PMN-MDSCs, Gr-1+CD11b+/−F4/80−; MO-MDSCs, Gr-1+CD11b+F4/80−) was ≥98%. RNA was extracted from sorted MDSC subsets using the RNeasy Mini Kit (Qiagen). Obtained RNA was treated with DNase using TURBO DNA-free kit (Ambion) and precipitated with linear polyacrylamide. Gene expression was determined using the Mouse Ref-8 v2 array from Illumina. Three samples generated in three independent experiments per group were analyzed. cDNA synthesis and hybridization to arrays were performed according to the manufacturer’s instructions. Raw microarray data were processed using BeadStudio v9. Briefly, raw values were quantile normalized, background subtracted, and log2 transformed. Statistical and cluster analyses were performed using the software package MeV v4.6 (MultiExperiment Viewer) from TM4 Microarray Software Suite. Statistical analysis of microarrays was used to identify significantly differentially expressed genes between defined groups. A false

Chemotaxis assay

Tregs from spleens of naïve C57BL6-CD45.1−/− mice or CCR5−/− mice were enriched by magnetic beads using CD4+CD25+ isolation kit (Miltenyi Biotec; purity ≥95%) and stimulated with 5 μg/ml anti-CD3/CD28 mAbs overnight in serum-free primary cell medium (RPMI with 1% L-glutamine, 1% penicillin, 1% streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 50 μM 2-ME). This preactivation increased the percentages of CCR5+ Tregs from 7 to 40% (data not shown). Tumor-infiltrating MDSC subsets from C57BL6 mice expressing CD45.2 were sorted by FACS and placed in 600 μl primary cell medium in the lower wells of an HTS Transwell-24 (Corning) with a 5-μm pore size. After 18 h, pre-activated Tregs (2 × 105 in 200 μl) were added to the upper chamber and cultured for 5 h. Migrated Tregs were harvested from the lower chamber and stained with anti-CD45.1 mAbs. After staining, Accudrop Counting Beads (BD Biosciences) were added, and the numbers of cells were determined by CD45.1 expression using the FACSCalibur flow cytometer (BD Biosciences). Purified Tregs from CCR5−/− mice were labeled with CFSE, and numbers of migrated cells were distinguished by CFSE labeling after addition of Accudrop Counting Beads (BD Biosciences).

Cell preparation and flow cytometry

Peripheral blood was collected, and erythrocytes were lysed. Tumors were removed, cut into pieces, and digested in 0.5 mg/ml hyaluronidase type V and 0.02% sodium azide for 30 min at 37˚C. Living tumor cells were enriched using Lympholite M gradients (Cedarlane Laboratories). Proteinases and 0.5 mg/ml DNase I (both Sigma-Aldrich) for 30 min at 37˚C. Living and the melanoma cell line B16BL6 (American Type Culture Collection, ATCC) were cultured in RPMI 1640 and DMEM, respectively, with 10% FCS, 1% l-glutamine, 1% penicillin, and 1% streptomycin (Invitrogen). RMA-S (5 × 106 or 106 as indicated) or B16 (5 × 105) cells were incubated into the right flank of mice. Tumors were measured every second or third day with a caliper, and the volume (length × width × height) was calculated.

In vivo BrdU staining

RMA-S tumor–bearing or naïve mice were injected with 2 mg BrdU i.p. on day 18 after tumor cell inoculation. After 1 h, mice were sacrificed and bone marrow, blood, spleen, and tumor suspensions were prepared. BrdU incorporation in MDSCs was analyzed using the BD FITC BrdU Flow Kit (BD Biosciences) according to the manufacturer’s instructions.

Chemokine application in vivo

A total of 1 μg CCL3, CCL4, or CCL5 (Peprotech) were coinjected with the tumor cells. Chemokines (1 μg/mouse) were reinjected intratumorally every third day. A total of 10 μg/mouse Met-RANTES (kindly provided by Amanda Proudfoot; Merck Serono Geneva Research Center) was injected i.p. daily for 3 wk.
discovery rate of 5% was used as a cutoff for determination of significance (Gene Expression Omnibus accession number GSE41620; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE41620).

Statistical analysis

Differences between groups were calculated by unpaired Student t test or by two-way ANOVA. Values of p < 0.05 were considered to be statistically significant.

Results

MDSC subsets accumulate in peripheral blood, spleen, and tumor tissue of RMA-S lymphoma–bearing mice

The aim of our study was to elucidate the molecular signature and function of MDSC subsets in the tumor tissue of mice. As a first step, numbers of MDSC subsets were determined in mice after s.c. inoculation of RMA-S lymphoma cells. Within 3 wk, progressive tumor growth was accompanied by an expansion of Gr-1+ and Gr-1+CD11b+ cells in peripheral blood and spleen (Fig. 1A). Higher amounts of CD11b+ cells were observed in spleen of tumor-bearing mice. Tumor-infiltrating cells consisted mainly of Gr-1+ and CD11b+ that comprised Gr-1+CD11b+ cells and Gr-1+CD11b+F4/80− cells that were previously described as tumor-associated macrophages (3). In addition, lower percentages of CD4+ T cells, Tregs, CD8+ T cells, and NK cells were present in the tumor tissue (26). Two MDSC subsets, Gr-1+CD11b+F4/80− and Gr-1+CD11b+F4/80+ cells, exhibited distinct morphological properties (Fig. 1B). Gr-1+CD11b+F4/80− MDSCs from blood and tumor were polymorphonuclear (PMN-MDSC), whereas Gr-1+CD11b+F4/80+ MDSCs displayed mononuclear features (MO-MDSC). Of note, PMN-MDSCs from blood and tumor expressed Ly6G and low levels of Ly6C, whereas MO-MDSCs displayed a Ly6G−Ly6Chigh phenotype (data not shown). To determine the kinetics of PMN- and MO-MDSC accumulation, total cell numbers of these subsets were assessed in blood and tumors at indicated time points (Fig. 1C).

In blood of tumor-bearing mice, both subpopulations accumulated during tumor progression, but the frequency of PMN-MDSCs was significantly higher. In tumor tissue, PMN- and MO-MDSCs accumulated with comparable kinetics, and similar cell numbers were detected. To address the generation/expansion of tumor-infiltrating MDSCs, we analyzed BrdU incorporation in MDSC subsets from blood, spleen, bone marrow, and the tumor tissue. BrdU+ PMN-MDSCs were only detected in the bone marrow at low percentages (Fig. 1D). High percentages of BrdU+ MO-MDSCs were mainly detected in the bone marrow regardless of whether naive or tumor-bearing mice were analyzed. In the tumor tissue, only 4% MO-MDSCs were labeled with BrdU, suggesting that their accumulation in the tumor tissue was mainly due to their recruitment from the bone marrow and to a lesser extent to proliferation at the tumor site.

Suppressive factors are produced by tumor-infiltrating MDSC subsets

To evaluate factors involved in suppression by tumor-infiltrating MDSC subpopulations, we determined the frequency of CD4+Foxp3+ Treg cells in blood and tumor of mice treated with s.c. inoculation of RMA-S–injected mice. MO-MDSC subsets displayed significantly higher arginase-1 and NO production as compared with their granulocytic counterparts (Fig. 2A 2B). Tumor-infiltrating MO-MDSCs showed dramatically (28-fold) higher levels of arginase-1 and increased levels (3-fold) of NO compared with blood MO-MDSCs. Furthermore, the PMN-MDSC subsets produced highly elevated levels of ROS compared with MO-MDSCs (Fig. 2C). Highest amounts of ROS were detected in PMN-MDSCs from blood. Overall, our results demonstrate high arginase-1 and inducible NO synthase (iNOS) activity by MO-MDSCs from solid tumors and ROS production by PMN-MDSCs, indicative of the suppressive function of tumor-infiltrating MDSC subsets.

Gene expression profiling reveals high chemokine expression in tumor-infiltrating MO-MDSCs

To further characterize tumor-infiltrating MDSC subpopulations, we performed genomewide expression profiling using Illumina microarray chips (Illumina). PMN- and MO-MDSCs were isolated from peripheral blood of naive C57BL/6 mice and from blood and tumor of RMA-S–tumor–bearing mice when the tumor diameter reached 1.5 cm (day 19 after tumor cell inoculation). To obtain highly purified MDSC subsets (>98%), cells were sorted by flow cytometry. mRNA samples obtained in three independently performed experiments were analyzed. Pan-genomic microarray analysis revealed a cluster of >400 genes that were explicitly upregulated in MO-MDSCs from tumor tissue compared with all other samples (Supplemental Table I). Moreover, 250 genes were significantly upregulated in tumor-infiltrating MO-MDSCs compared with MO-MDSCs from blood of tumor-bearing mice (Supplemental Table II). Among the highly differentially expressed genes (fold change >5), we detected several members of the chemokine family, including the chemokines Ccl3, Ccl4, Cxcl11, Cxcl9, Cxcl2, Ccl13, and Ccl4 (Fig. 3A, 3B). In the tumor-infiltrating PMN-MDSC subset, only CCL3 was significantly increased. Because the genes Ccl3 and Ccl4, encoding the CCR5 ligands CCL3 (MIP-1α) and CCL4 (MIP-1β), showed the strongest upregulation, protein levels of all CCR5 ligands were analyzed. Indeed, tumor-infiltrating MO-MDSCs produced significantly higher levels of CCL3, CCL4, and CCL5 compared with all other subsets (Fig. 3C). The RMA-S cell line neither produced any of these chemokines nor expressed CCR5 (data not shown). Moreover, tumor-infiltrating MO-MDSCs from B16 melanoma were the most potent producers of CCL3, CCL4, and CCL5 among PMN- and MO-MDSC subsets isolated from blood and tumor (Fig. 3D). Finally, we analyzed MDSC subsets in a ret transgenic mouse model of spontaneous skin melanoma. In this mouse model, after a short latency, ~25% mice develop microscopic skin melanoma with disseminated metastases in lymph nodes, bone marrow, lung, liver, and brain (25, 27). As shown in Fig. 3E, highly elevated levels of CCL3, CCL4, and CCL5 were detected in MO-MDSCs from the peripheral blood of mice carrying skin melanoma as compared with nontransgenic littermates. Lower amounts of CCL3, CCL4, and CCL5 were detectable within the tumor-infiltrated MO-MDSCs.

Chemotraction of Tregs to the tumor tissue by CCL4 and CCL5

So far, our results indicate that CCL3, CCL4, and CCL5 were the most abundant chemokines produced by tumor-infiltrating MO-MDSCs. The only chemokine receptor shared by all of the three chemokines CCL3, CCL4, and CCL5 is CCR5. CCL3 and CCL5 also bind to other receptors. To determine the cell population that might be recruited to the tumor tissue via CCR5, immune cells from blood, spleen, and tumor were stained for CCR5 surface expression. On day 17 after tumor cell inoculation, Tregs from peripheral blood and spleen of tumor-bearing mice and from tumor tissue displayed elevated levels of CCR5, whereas expression of CCR5 was hardly detectable on conventional CD4+Foxp3+ T cells (Fig. 4A, B). CD8+ T cells from blood and spleen expressed CCR5 at low levels and expression of CCR5 was increased on tumor-infiltrating CD8+ T cells.

Because Tregs exhibited high surface expression of CCR5, we investigated whether CCL3, CCL4, or CCL5 attracts Tregs to the tumor tissue in vivo. Upon intratumoral injection of recombinant
chemokines, we observed a significant increase of tumor-infiltrating Tregs among CD4+ T cells after CCL4 and CCL5 (Fig. 4C). The injection of CCL3 did not significantly alter the percentage of Tregs in the tumor tissue. Taken together, these results demonstrate that the CCR5 ligands CCL4 and CCL5 promote the accumulation of Tregs within the tumor tissue.

**Tumor-infiltrating MO-MDSCs recruit Tregs via CCR5**

To validate the chemoattractive potential of tumor-infiltrating MDSC subsets, Treg migration toward tumor-infiltrating MDSCs was analyzed in vitro. As shown in Fig. 5A, high numbers of Tregs from CCR5 knockout (KO)
mice almost completely lost the ability to migrate toward chemokines secreted by MO-MDSCs (Fig. 5A). Of note, similar development of Tregs in CCR5-deficient and wt mice was described (28).

**CCR5 deficiency and CCR5 blockade result in delayed tumor growth**

To analyze the effect of CCR5 deficiency in vivo, the amounts of tumor-infiltrating conventional CD4+Foxp3+ CD8+ T cells, and Tregs from wt or CCR5-deficient mice were assessed. Analysis of lymphocyte populations revealed significantly reduced levels of Tregs in the tumor tissue of RMA-S tumor-bearing CCR5-deficient mice (Fig. 5B). Furthermore, higher ratios of CD4/Tregs and CD8/Tregs were detected in tumors of CCR5 KO mice compared with wt mice. Decreased Treg numbers were also detected at late stage tumors (day 19) from CCR5 KO mice (data not shown). Although CCR5 was expressed by MO-MDSC from blood and tumor, similar amounts of tumor-infiltrating MO-MDSC were observed in CCR5 KO and wt mice (data not shown). The lower percentages of Treg correlated with reduced RMA-S tumor growth in CCR5 KO mice (Fig. 5C). In a next step, we analyzed mice bearing B16 melanoma. In B16 tumors, Treg levels in CCR5 KO mice were significantly reduced accompanied by an increased CD8/Treg ratio (Fig. 5D). In this tumor model, no differences in the CD4/Treg ratio between CCR5 KO and wt mice were detected. Similar to the RMA-S tumor model, tumor growth of B16 in CCR5 KO mice was significantly reduced compared with wt mice (Fig. 5E).

Finally, we investigated the effect of Met-RANTES, a CCL5 analog that inhibits agonist-induced activities, in wt mice after inoculation of different doses (1 × 106 or 0.5 × 106) of RMA-S tumor cells. In mice that received Met-RANTES, RMA-S tumor growth was significantly delayed compared with PBS-injected control mice regardless of whether 1 × 106 or 0.5 × 106 RMA-S cells were injected (Fig. 5F). Overall, our data indicate that CCR5 deficiency and CCR5 blockade result in delayed tumor growth.

**Discussion**

Tumors use a variety of suppressive strategies to escape from antitumor immune responses. One common feature of tumor progression is the deregulation of myelopoiesis and the expansion of MDSCs. MDSCs are a heterogeneous population comprising monocytic and granulocytic cells. The characterization of MDSC subsets present in the tumor tissue is crucial to understand their involvement in tumor-associated immune suppression. Thus, in our study, we investigated the molecular signature, phenotype, and function of the monocytic and granulocytic MDSC subsets isolated from tumors of RMA-S lymphoma–bearing mice compared with MDSCs from blood of tumor-bearing or naive mice.

A previous study described that Gr-1+CD11b+ MDSCs infiltrating 3LL tumors were composed primarily of macrophages and neutrophils (29). Moreover, Movahedi et al. (4) demonstrated that mouse splenic Gr-1+CD11b+ MDSCs comprised an Ly6G SSChigh polymorphonuclear (PMN) and an Ly6G+ SSClow mononuclear (MO) subset (4). MO-MDSCs expressed the macrophage marker F4/80 that was absent on PMN-MDSCs. In concordance with previous reports (4, 5, 30) we observed that PMN-MDSCs from blood and tumor expressed Ly6G, low levels of Ly6C, whereas MO-MDSCs displayed a Ly6G+ Ly6Chigh phenotype. Both populations expressed CD124 (31, data not shown). In our study, we characterized Gr-1+CD11b+ F4/80− PMN-MDSC and Gr-1+ CD11b+ F4/80+ MO-MDSC subsets in blood and tumor tissue. Our results reveal that both subpopulations increased in numbers during tumor growth in blood, spleens, and tumors. PMN-MDSCs represented the most prominent subset in the blood, whereas accumulation of MO-MDSCs was more pronounced in the tumor tissue.

Our experiments, addressing the proliferation of the two MDSC subpopulations by BrdU labeling, revealed a high turnover rate of MO-MDSCs in the bone marrow and only low percentages of BrdU+ MO-MDSCs in spleen and tumor. These data suggest that MO-MDSCs expand in the bone marrow and migrate through the blood stream to spleen and tumor, where they proliferate at low levels. Comparatively low percentages of BrdU+ PMN-MDSCs were detected in the bone marrow but not in the peripheral blood, spleen, or tumors, suggesting that these cells might migrate from the bone marrow to the tumor tissue and/or might arise from precursors other than Gr-1+CD11b+ F4/80+. These results are in line with a recent study addressing the turnover of MDSC subpopulations in the 3LL tumor model (29).

Several tumor-derived factors such as GM-CSF (32), Cox-2 (33), S100A8/A9 (34), IL-1β (35), or IFN-γ (4) mediate expan-
sion and activation of MDSC subsets and induce their suppressive activity (36). Our data indicate that MDSC subpopulations from blood and tumors differ in their capacity to produce factors involved in T cell suppression. PMN-MDSCs exhibited high levels of ROS in blood, lower levels in the tumor tissue, and negligible arginase-1 or iNOS activity. In contrast, tumor-infiltrating MO-MDSCs displayed increased amounts of arginase-1 and low levels of ROS. In our study, MDSC subsets were stimulated with LPS for NO production. Only tumor-infiltrating MO-MDSC produced high amounts of NO. In previous studies, similar levels of NO were induced upon stimulation with IFN-γ (37). The IFN-γ-induced iNOS expression was further increased upon addition of IL-13 (38). Thus, it is likely that also other more physiological stimuli such as IFN-γ/IL-13 would induce NO from tumor-infiltrating MO-MDSCs. Corzo et al. (39) demonstrated rapid upregulation of arginase-1 and iNOS in i.p. CD11b+Gr-1+ MDSCs in a tumor model of EL-4 lymphoma growing in the peritoneum. The upregulation of iNOS and arginase-1 and the decreased levels of ROS were mediated by the hypoxia-inducible factor-1α. Moreover, other transcription factors like STAT1, STAT6, or C/EBPβ were reported to be involved in the regulation of MDSC activity (40–42). The transcription factors mediating the sup-

**FIGURE 3.** Tumor-infiltrating MO-MDSCs express high levels of chemokines including the CCR5 ligands CCL3, CCL4, and CCL5. (A) Differentially expressed genes of the chemokine family were identified by microarray analysis on the different subsets of MDSCs. The heat map represents selected members of the chemokine family significantly upregulated in the tumor-infiltrating MO-MDSC subset. Expression is displayed as log2 intensity value generated from normalized microarray data. (B) Log2 intensity values of chemokine gene expression for Ccl3, Ccl4, Cxcl2, Cxcl9, Ccl7, and Ccl24 in different MDSC subsets are depicted. (C) MDSC subsets were FACS-sorted 19 d after RMA-S cell inoculation (pooled, n = 15) and cultured in vitro for 24 h. Supernatants were analyzed for chemokine production by Bioplex (Bio-Rad). Data are representative of three independent experiments. (D) B16 tumor cells were s.c. injected into mice. MDSC subsets from blood and tumor (pooled, n = 15) were purified on day 17 after tumor cell inoculation, and chemokine production was measured as described in (C). (E) MDSC subsets from the peripheral blood and skin tumors of ret transgenic mice (pooled n = 20) were purified, and the chemokine production was measured as described in (C). Graphs (B–E) represent the mean ± SEM of cultures set up in triplicates. MO, MO-MDSC; PMN, PMN-MDSC.
pressive activity of MDSCs in our model are currently under investigation.

Increasing evidence exists that chemokines recruiting inflammatory cells to the tumor tissue are produced not only by cancer cells, but also by tumor-infiltrating cells (43). In our study, global gene expression profiling of MDSC subsets from blood and tumor revealed distinct chemokine patterns. Purified MO-MDSCs isolated from the tumor tissue of RMA-S lymphoma- or B16 melanoma-bearing mice secreted high amounts of chemokines including CCL3, CCL4, and CCL5. To address the chemokine production by MO-MDSCs in a spontaneous autochthonous mouse model, skin cancer–bearing ret transgenic mice were analyzed. Highly increased levels of CCL3, CCL4, and CCL5 were found in MO-MDSCs from blood. Lower levels of these chemokines were detected in MO-MDSCs from tumors. It is likely that in this model disseminated melanoma metastases also affected MO-MDSCs in the blood. Of note, other myeloid cells present in the tumors such as Gr-1$^+$ tumor-associated macrophages also produced certain chemokines (data not shown). The chemokine production by non-MDSC myeloid subsets and their function in the tumor microenvironment will be addressed in future studies. Previously, it has been reported that MDSCs can produce certain chemokines (44), but the chemokine production by MDSC subsets from different organs and the impact of MDSC-derived chemokines on the recruitment of immune cells to the tumor tissue has not been addressed so far. In this study, we show for the first time, to our knowledge, that chemokine secretion by tumor-infiltrating MO-MDSCs results in direct Treg recruitment dependent on CCR5.

CCL5–CCR5 interactions have already been linked to the tumor microenvironment (45). However, several aspects of CCR5 and its ligands in cancer have remained unclear. In concordance with our results indicating a protumoral role of CCR5/CCR5 ligands in cancer, CCL5 secretion by mesenchymal stem cells enhanced the metastatic potential of breast cancer (46). Ng-Cashin et al. (47) demonstrated that the deficiency of CCR5 or its ligand CCL3 resulted in delayed or accelerated melanoma growth depending on the tumor load. Furthermore, in a mouse model of pancreatic adenocarcinoma, knockdown of CCL5 production in tumors resulted in delayed or accelerated melanoma growth depending on the tumor load. In contrast, CCL5/CCR5 blockade improved the efficiency of immunochemotherapy (48). In our models, tumor-infiltrating CD8$^+$ T cells expressed CCR5 but their accumulation in tumors in CCR5-deficient mice was higher compared with wt mice, indicating that CCR5 expression was not required for their tumor infiltration. In contrast, CCR5-deficient mice exhibited lower numbers of CD4$^+$ and CD8$^+$ T cells and similar amounts of Tregs infiltrating pancreatic carcinoma compared with wt mice (49). The diverging tumor-promoting versus tumor-delaying results described for CCR5/CCR5 ligands might be explained by the use of different tumor models. We believe that the differential ability of tumor cells to produce certain chemokines by themselves, the immunogenicity of tumor cells, and the types and amounts of tumor-infiltrating immune cells greatly influence the contribution of the CCR5/CCR5 ligand axis to the outcome of antitumor responses.

Previously, we have shown that depletion of Tregs in RMA-S lymphomas increased antitumor immunity mediated by NK and...
T cells, supporting the suppressive role of Tregs in our model (26). Because Tregs are able to suppress T cells at the tumor site (11) and their accumulation in tumors correlates with bad prognosis (16), the identification of factors involved in Treg accumulation in tumors is of high clinical relevance. Our results demonstrate that tumor-infiltrating MO-MDSCs directly mediate the migration of Tregs via CCR5 in vitro. In vivo, deficiency of CCR5 resulted in reduced Treg numbers in the tumor tissue in RMA-S and B16 tumor–bearing mice. Increased CD4/Treg ratios were only detected in RMA-S tumors and not in B16 tumors from CCR5 KO mice. It is likely that in B16 tumors, the composition of CD4+ T cells within the tumor tissue differs from RMA-S tumors due to the different tumor microenvironment. At this point, we cannot formally exclude that in addition to CCR5, other mechanisms are also directly involved in recruitment of Tregs to tumors in vivo.

Collectively, we demonstrate for the first time, to our knowledge, that the monocytic subset of tumor-infiltrating MDSCs is a potent producer of chemokines resulting in CCR5-dependent Treg re-

FIGURE 5. Tumor-infiltrating MO-MDSCs attract Tregs in a CCR5-dependent manner. (A) Tumor-infiltrating MDSC subsets were purified by FACS on day 19 after tumor cell inoculation (pooled, n = 30) and 5 \times 10^5 (left panel) and 4 \times 10^5 cells (right panel) were cultured for 18 h in the lower chamber. Preactivated Tregs from naive C57BL/6 wt (pooled, n = 6) or CCR5 KO mice (pooled, n = 6) were added to the upper chamber, and the percentage of migrated Tregs was assessed after 5 h. Values represent the mean (left panel, n = 3; right panel, n = 2) \pm SEM. (B) Amounts of immune cells in the tumor tissues of wt or CCR5 KO mice were analyzed on day 11 after RMA-S tumor cell inoculation. Percentages of Tregs within total CD4+ T cells and the CD4/Treg and CD8/Treg ratios are depicted. (C) wt and CCR5-deficient mice were s.c. injected with RMA-S cells. Tumor growth was assessed for 19 d. (D) Amounts of immune cells in the tumor tissues of wt or CCR5 KO mice were analyzed on day 14 after B16 tumor cell inoculation. Percentages of Tregs within total CD45+ cells and the CD4/Treg and CD8/Treg ratios are depicted. (E) wt and CCR5-deficient mice were s.c. injected with B16 cells. Tumor growth was assessed for 14 d. (F) C57BL/6 mice were s.c. injected with RMA-S cells and i.p. with 10 \mu g Met-RANTES or PBS every day. Tumor growth was determined as indicated. Data are representative of three independent experiments. Values (B–F) represent the mean (B, n = 4; C, n = 5; D, E, n = 12; F, n = 9) \pm SEM. *p < 0.05, **p < 0.01.
suppression and T regulatory cell activation mediated by myeloid-derived suppres-
CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1
sor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory
Competition of transpant tolerance and intragraft regulatory T cell localization by
E. Suri-Payer, and A. Cerwenka. 2010. Regulatory T cells control macrophage
2008. Mononuclear myeloid–derived “suppressor” cells express RA-E1 and activate
High-dose granulocyte-macrophage colony-stimulating factor-producing vac-
cines improve the immune response through the recruitment of myeloid suppressor
29. Rodriguez, P. C., C. P. Hernandez, D. Quiceno, S. M. Dubinett, J. Zabaleta,
regulates function and
expression of myeloid-suppressor cells and induction of M1 macrophages facilitate the
2008. Identification of discrete tumor-inducified myeloid-suppressor cell sub-
32. Marigo, I., L. Dolcetti, P. Serafini, P. Zanovello, and V. Bronte. 2008. Tumor-
induced tolerance and immune suppression by myeloid-derived suppressor cells.
33. Ostrand-Rosenberg, S., and P. Sinha. 2009. Myeloid-derived suppressor cells:
34. Bronte, V., and P. Zanovello. 2005. Regulation of immune responses by L-
35. Serafini, P., J. P. Chen, T. Kroemer, A. H. Banham, M. Hellstrom, L. Egevad,
and P. Pisa. 2006. CD4+CD25high T cells are enriched in the tumor and peripheral
blood of prostate cancer patients.
36. Song, X., Y. Krelin, T. Dvorkin, O. Bjorkdahl, S. Segal, C. A. Dinarello,
203: 2451–2460.
High-dose granulocyte-macrophage colony-stimulating factor-producing vac-
cines improve the immune response through the recruitment of myeloid suppressor
38. Rodriguez, P. C., C. P. Hernandez, D. Quiceno, S. M. Dubinett, J. Zabaleta,
regulates function and expression of myeloid-suppressor cells and induction of M1 macrophages facilitate the
39. Gallyna, G., L. Dolcetti, P. Serafini, C. De Santo, A. Fernandez, L. Dolcetti,
cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting
40. Ribechni, E., V. Greifenberg, S. Sandwick, and M. B. Lutz. 2010. Subsets,
Myeloid-derived suppressor cell activation by combined LPS and IFN-gamma treat-
42. Marigo, I., E. Bosio, S. Solito, C. Mesa, A. Fernandez, L. Dolcetti, S. Ugel,
cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting
43. Kusmartsev, S., and D. I. Gabrilovich. 2005. STAT1 signaling regulates tumor-
45. Soria, G., E. A. Fein, S. Solito, C. Mesa, E. Fernandez, L. Dolcetti, S. Ugel,
550.
47. Uozumi, N., M. Tsuji, T. Yonemochi, H. Kitoh, B. Kain, T. Nonaka, G. F. Ouyang,
suppressor cells express RAE-1 and activate natural killer cells. J. Immunol.
180: 6174–6184.
Y. Yin, C. M. Divino, and S. H. Chen. 2010. Immune stimulatory receptor CD40 is required for T-cell

