Complement 5a Enhances Hepatic Metastases of Colon Cancer via Monocyte Chemoattractant Protein-1-mediated Inflammatory Cell Infiltration*

Chunmei Piao‡§, Lun Cai†, Shulan Qiu‡, Lixin Jia‡, Wenchao Song‡, and Jie Du‡§†

From the ‡ Beijing Anzhen Hospital Affiliated to the Capital Medical University, Beijing 100029 and † The Key Laboratory of Remodeling-Related Cardiovascular Diseases, Capital Medical University, Ministry of Education, Beijing Institute of Heart Lung and Blood Vessel Diseases, Beijing 100029, China

**Background:** It is known that the complement system contributes to tumor progression, but the exact mechanism is still unclear.

**Results:** Complement 5a enhances tumor metastasis via monocyte chemoattractant protein-1-mediated inflammatory cell infiltration.

**Conclusion:** Complement 5a plays a pro-metastasis role by establishing an inflammatory microenvironment required for tumor metastasis.

**Significance:** Our results provide a therapeutic insight for complement in treatment of malignant tumors.

Complement 5a (C5a), a potent immune mediator generated by complement activation, promotes tumor growth; however, its role in tumor metastasis remains unclear. We demonstrate that C5a contributes to tumor metastases by modulating tumor inflammation in hepatic metastases of colon cancer. Colon cancer cell lines generate C5a under serum-free conditions, and C5a levels increase over time in a murine syngeneic colon cancer hepatic metastasis model. Furthermore, in the absence of C5a receptor or upon pharmacological inhibition of C5a production with an anti-C5 monoclonal antibody, tumor metastasis is severely impaired. A lack of C5a receptor in colon cancer metastatic foci reduces the infiltration of macrophages, neutrophils, and dendritic cells, and the role for C5a receptor on these cells were further verified by bone marrow transplantation experiments. Moreover, C5a signaling increases the expression of the chemokine monocyte chemoattractant protein-1 and the anti-inflammatory molecules arginase-1, interleukin 10, and transforming growth factor β, but is inversely correlated with the expression of pro-inflammatory molecules, which suggests a mechanism for the role of C5a in the inflammatory microenvironment required for tumor metastasis. Our results indicate a new and potentially promising therapeutic application of complement C5a inhibitor for the treatment of malignant tumors.

Malignant tumors are characterized by their ability to metastasize. Increasing evidence shows that the development of a supportive microenvironment in solid tumors plays a critical role in tumor metastasis (1). In the tumor microenvironment, inflammatory cells and molecules influence almost every aspect of cancer progression, including tumor metastasis (2). Inflammatory immune cells constitute a substantial proportion of the cells within the tumor microenvironment and are associated with tumor malignancy and animal models of cancer (3). It is clear that the immune system is a major contributor to pathogenesis, although the mechanisms of tumor metastasis are not fully understood. Therefore, a better understanding of the underlying immune-mediated pathways involved in tumor metastasis may identify new targets that could be manipulated pharmacologically or biologically to halt disease progression.

As a central contributor to the innate immune response, complement plays a major role as a first defense against harmful molecules and microbes that are unwanted by the host (4–6). Anaphylatoxins (complement 3a (C3a), complement 4a (C4a), and complement 5a (C5a)) are a group of small peptides generated by complement activation that play important roles in innate immunity through the initiation and regulation of inflammatory responses (7, 8). Complement activation contributes to cancer progression, and complement deposition is observed in different tumor types (9–11). However, the function of C5a in tumor metastasis is controversial (12). In some studies complement shows an active and beneficial role in the fight against malignant cells, and complement-dependent cytotoxicity synergizes with tumor-directed antibody therapy in tumor treatment (13, 14). Markiewski et al. (15) demonstrated that C5a in the tumor microenvironment leads to significant tumor progression in a mouse model of cervical cancer, which is mediated, in part, by the recruitment of myeloid-derived suppressor cells. Furthermore, lung cancer cells can produce complement C5a, and blocking C5a by antagonist inhibited tumor growth (16). These findings suggest that C5a contributes to tumor growth in the immunosuppressive microenvironment. Complement activation may also be linked to angiogenesis. In human colon cancer, the immune response strongly influences tumor metastasis (17), and elevated complement levels in...
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hepatic metastases are observed in colon cancer patients (18). Sixty percent of patients with colon cancer develop liver metastasis, which is responsible for a large percentage of colon cancer-related deaths (19, 20). However, the function of C5a in hepatic metastasis of colorectal cancer has not been elucidated. Therefore, we sought to demonstrate C5a function with emphasis on the tumor microenvironment.

In this context, we hypothesized that complement activation may contribute to the generation of an inflammatory microenvironment that favors colon cancer metastasis. Our results demonstrate that C5a is released and promotes a pro-tumor environment through a mechanism that involves increased inflammatory infiltration, the production of monocyte chemoattractant protein-1 (MCP-1),2 and a reduction in the levels of immune modulators. These results provide new information about the relationship between complement activation and tumor metastasis, which could influence the development of future therapeutic strategies.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The antibody against Ki-67 was from Santa Cruz Biotechnology (Santa Cruz, CA); the antibodies against F4/80, C5a receptor, and Ly6G were from Abcam (Cambridge, MA); and ChemMate™ EnVision System/DAB Detection Kits were from Dako (Glostrup, Denmark). The following antibodies were from Biolegend (San Diego, CA): PerCP/Cy5.5-conjugated CD45.2, phycoerythrin-conjugated F4/80, fluorescein isothiocyanate (FITC)-conjugated F4/80, FITC-conjugated CD206, FITC-conjugated CD4, FITC-conjugated CD8, and isotype controls. Anti-mouse C5 monoclonal antibody (BB5.1) and the irrelevant IgG control of the same isotype (MOPC), which is a widely used C5 blocking antibody, and control antibody had been previously demonstrated for its effectiveness were used as described previously (21–23). Protein kinase B (also known as Akt) inhibitor MK-2206 was from Selleck Chemicals (Huston, TX). Recombinantly mouse C5a was from R&D Systems (Minneapolis, MN). Mouse C3a and C5a ELISA Kits were from KeYingMei Technology Co. Ltd. (KYM, Beijing, China).

Cell Culture—SL4 colon carcinoma cells were maintained in DMEM/F-12 culture medium as described (24), HCT116 human colorectal carcinoma cells and SW480 human colon adenocarcinoma cells were maintained in Iscove’s modified Dulbecco’s medium, CT26 mouse colon cancer cells were maintained in RPMI 1640 medium. Cultures were supplemented with 10% fetal bovine serum (FBS) and 100 units/ml each penicillin and streptomycin and grown under a 5% CO2 at 37 °C. All cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Animals—C5aR−/− mice, backcrossed onto the genetic background of C57BL/6 for more than 10 generations, were as described previously (14). Mice were 8–12 weeks old at the beginning of the experiments and were matched for age and sex with wild-type (WT) mice. All mice were housed under specific pathogen-free conditions at the Beijing Anzhen Hospital, which is affiliated with the Capital Medical University, China. All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People’s Republic of China (documentation number 55, 2001) and the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (publication number 85-23, revised 1996) and approved by the Institutional Animal Care and Use and Committee of the Capital Medical University.

Tumor Model and Administration of Anti-C5 mAbs—SL4 colon carcinoma cells were derived from C57BL/6 mice on the same background as the C5aR−/− and WT control mice, as described previously (25). For the in vivo hepatic metastasis model, after anesthetizing mice, a transverse incision in the left flank was made, exposing the spleen, and then 5 × 105 SL4 tumor cells in 100 μl of DMEM/F-12 medium were intrasplenically injected using a 26-gauge needle. Hepatic ultrasonography was performed on mice at day 14 after SL4 injection using a 15-MHz probe for adults and the VEVO 770 software package (Visual Sonics). Fourteen days after inoculation, mice were sacrificed, and the tissues were processed as described below. The spleen and liver were removed, wet spleen and liver weights were measured, and the incidence of hepatic metastases was examined. The neutralizing monoclonal anti-C5 antibody BB5.1 or isotype control mouse IgG1, MOPC-31C (all at a dose of 1 mg/mouse), were administered as described previously (23).

Histology and Immunohistochemistry—Paraffin serial sections of 5 μm thick were obtained for histologic analysis, and stained with hematoxylin & eosin (HE) by standard procedures (26). Paraffin sections were incubated with the primary antibody against Ki-67 (1:200) at 4 °C overnight and then incubated with the Dako ChemMate™ EnVision System (Dako, Glostrup, Denmark) for 30 min. Images were viewed and captured using a Nikon Labophot 2 microscope equipped with a Sony CCD-IRIS/RGB color video camera attached to a computerized imaging system and analyzed by Image Pro Plus 3.0. The expression of Ki-67 was calculated as the proportion of positive area to total tissue area for all measurements of the sections, using the Aperio (Vista, CA) Full Automatic Digital Slide Scanning System (27).

Frozen tumor sections (7 μm) and cell slides were incubated with the primary antibodies against F4/80 (1:100), C5aR (1:200), or Ly6G (1:200), at 4 °C overnight and then with FITC- or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) at room temperature for 1 h. Sections were viewed with a confocal laser scanning microscope (TCS 4D; Leica, Heidelberg, Germany) and a Nikon Labophot 2 microscope equipped with a Sony CCD-IRIS/RGB color video camera (28).

SL4 sh-C5 Cells Selection—SL4 cells were transfected with a plasmid expressing C5 shRNA from Santa Cruz Biotechnology to down-regulate C5, and puromycin (1 μg/ml) were used for screening the stably transfected cell colonies. Reduction in C5 was confirmed by real-time PCR with primers for C5 shown in Table 1.

2The abbreviations used are: MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory proteins 1α; MIP-1β, macrophage inflammatory proteins 1β; RANTES, regulated on activation, normal T cell expressed and secreted; BM, bone marrow; C5aR, receptor for C5a.
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**Table 1**

Sequences of primers used in real-time PCR

| Primer | Forward | Reverse |
|--------|---------|---------|
| MCP-1  | CAG GTC CCT GTC AGT CTT CT | GTC AGC ACA GAC CTC TCT CT |
| MIP-1α | CCA AGT CTT GTC AGC GCC AT | GAT GAA TGG GGG GTG AAT CTT C |
| MIP-1β | TTC TCG TGG CTT CCT CT | TGC CGG GGA GGT GTA AGA GA |
| MIP-1γ | CCC CTC TTCC TCTCTAT TTCC TACA | AGCTTGAAGGCCCAGTGGAA |
| RANTES | ACC ATG AAC ATC TCT GCA GC | TGGCAAGATGTCGAGGAGG |
| Arginase | CCGGAGGACACTGAGGAGAGA | CACGCGACGACTGAGGAGAG |
| IL-1β | GCT TAC AAT GTG ATA GTC AAC | TTTTCACAGGGGAGAAATCG |
| IL-6 | CCG TCC ACA AAG TAT AAT AC | CACGCGACGACTGAGGAGAG |
| IL-10 | CCA GCT TTT ATG CAG GAA A | TTTCACAGGGGAGAAATCG |
| IL-12p35 | CCG CAC GAG CAT CTT TCC CAA | TGGGACAGACGAGACGAG |
| IL-12p40 | GAG TCC CAG CTC CAG GGG GCA | TGGGACAGACGAGACGAG |
| IL-23p1 | TCC TAC TAC GACT CCC ACA | TGGGACAGACGAGACGAG |
| TGFβ | TGG TTA CAG CAC GAG AAG | TGGGACAGACGAGACGAG |
| Nitric-oxide synthase 2 | GGC GCT TCC AGG GAT CTA | CAC GAG TGG TCC TCT GAG |
| Tubulin | TTC AAC CCG GGC TGC TCA | GCC GAG TGG TCC TCT GAG |
| C5-1 | GCA AG AAG AGA CAC GCT GAA | TCC GTC TCA GAG GTC TCA |
| C5-2 | GCA AAG AGA GAG CAT TCC CCA | TGG GGA GGT GGT CTA GAG |

**Apoptosis Analysis**—The apoptosis of formalin-fixed liver sections from WT or C5aR−/− mice after SL4 cell inoculation was analyzed by the TdT-mediated dUTP Nick-end Labeling (TUNEL) technique from Promega (Madison, WI).

**Macrophage and Neutrophil Extraction, Culture, and Treatment**—Treated mice were injected with 1.5 ml of 3.85% thioglycolate 3–5 days before macrophage isolation, 3–4 h before neutrophil isolation. Peritoneal macrophages and neutrophils were obtained by lavaging the peritoneal cavity with 5 ml of 10 mM phosphate-buffered saline (PBS), cells underwent hemocytometry and plating in 6-well plates at 3 × 10⁵ cells per well, then culture in DMEM containing 10% fetal bovine serum. After 4 h, non-adherent cells were removed by changing the medium (29). For recombinant C5a treatment, cells were washed with PBS and incubated with recombinant C5a (10 nm) for 6 h.

**Flow Cytometry**—For staining of immune markers, single-cell suspensions were prepared by mechanical dispersion and enzymatic digestion of tumor tissues. Briefly, tumor tissues were cut into multiple small cubes and digested in an enzyme mixture containing collagenase type I (0.05 mg/ml) and type IV (0.05 mg/ml), hyaluronidase (0.025 mg/ml), DNase I (0.01 mg/ml), and soybean trypsin inhibitor (0.01 mg/ml) for 45 min at 37 °C. The cell suspension was centrifuged and preincubated with fragment crystallizable blocking antibody (anti-mouse CD16/32; PharMingen, San Diego, CA) to prevent nonspecific binding. Cell staining involved different combinations of fluorochrome-coupled antibodies to CD45.2, F4/80, CD11b, Ly6G, CD11c, CD3, and CD8 for 30 min at 4 °C in the dark. Fluorescence data were collected using an EPICS XL Flow Cytometer (Beckman Coulter, Fullerton, CA), and analyzed using CellQuest. A fluorescence-negative control was included to determine the level of nonspecific staining and autofluorescence associated with subsets of cells in each fluorescence channel (30).

**Bone Marrow Transplantation**—Bone marrow was harvested from 6–8-week-old WT and C5aR KO mice by flushing the femurs and tibias with 2% FBS in PBS. Cells (2 × 10⁶) were intravenously injected through the tail vein of lethally irradiated (10 gray) recipient mice. Tumor cell implantation was performed by intrasplenic injection 8 weeks later.

**Real-time PCR**—Total ventricular RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. PCR amplification was performed using the iQ5 Real-time PCR Detection System (Bio-Rad) with SYBR Green Jump-Start™ Taq ReadyMix™ (Takara, Otsu, Shiga, Japan) and primers for mouse, MCP-1, macrophage inflammatory proteins 1α (MIP-1α), macrophage inflammatory proteins 1β (MIP-1β), macrophage inflammatory proteins 1γ (MIP-1γ), regulated on activation, normal T cell expressed and secreted (RANTES), arginase 1, interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12)-p35, IL-12-p40, interleukin 23 (IL-23)-p19, transforming growth factor β1 (TGFβ1), and nitric-oxide synthase 2 (Table 1). Melting curve analysis was performed at the end of each PCR. The housekeeping gene α-tubulin was used as control, and the expression of other genes was expressed as a ratio to the expression of α-tubulin.

**Chemokine Analysis**—To evaluate the production of chemokines in macrophages and neutrophils, mouse peritoneal macrophages and neutrophils were plated at 1 × 10⁶ per well in 24-well plates and cultured for 6 h. After incubation, the medium was collected and analyzed using BD LSR Fortessa and FACSDiva software and BD™ Cytometric Bead Array (CBA) kits (BD Biosciences) to measure levels of the chemokine keratinocyte chemoattractant, MCP-1, MIP-1α, MIP-1β, and RANTES.

**Western Blotting**—Protein extracts were diluted with loading buffer and separated by electrophoresis on 10% SDS-polyacrylamide gels before transfer to nitrocellulose membranes. The membranes were blocked in Odyssey blocking buffer (LI-COR Bioscience, Lincoln, NE) at room temperature for 2 h, then incubated at 4 °C overnight with primary antibodies: phospho-p38, p38, phosphor-Akt, Akt, phosphor-p42/44, p42/44, or actin (1:1000) (Cell Signaling Technology, MA). The membranes were washed and incubated with fluorescent secondary antibodies (Alexa Fluor 680 or IRDye 800, Rockland Immunochemicals, PA) for 1 h at room temperature at 1:5000; blots were analyzed with the Odyssey infrared imaging system and Odyssey software (31).
the bottom chamber. After 24 h, noninvading cells were removed from the upper membrane surface of the insert using a cotton swab. The invading cells on the lower membrane were stained and counted (32).

Statistical Analysis—Results are expressed as mean ± S.E. Data analysis involved the use of GraphPad software (GraphPad Prism version 5.00 for Windows). Comparison between groups was analyzed by one-way analysis of variance; *p < 0.05 versus time 0. B, generation of C5a by mice colon cancer SL4 or CT26 cells after 48 h incubation in serum-free medium. C, generation of C5a by human colon cancer HCT116 or SW480 cells after 48 h incubation in serum-free medium. D, plasma C5a levels over a time course after intrasplenic injection of SL4 colon carcinoma cells and non-transformed NIH3T3 cells (5 × 10^5) into mice.

RESULTS

Colon Cancer Cells Produce C5a—The complement system can recognize tumor cells, and complement deposition is observed in different types of tumors (9, 15). Cells from some tumor types under serum-free conditions will activate and release C5a. To determine whether the colon cancer cell line SL4 releases C5a, we cultured SL4 cells in serum-free medium for 48 h. Conditioned medium was collected and C5a and C3a levels were determined (Fig. 1A). C5a levels were elevated, whereas C3a levels remained constant. C5a production in other mouse colon cancer cell lines and in human colon cancer cells were also high (Fig. 1B and C). To verify these results, we employed the SL4 syngeneic metastasis model of colon cancer in mice. In this model, metastatic foci in the liver develop after intrasplenic injection of colon cancer cells. Our results demonstrate that levels of C5a in the plasma are increased at 10 days after intrasplenic injection of colon cancer cells and remain high for at least 2 additional weeks, to rule out a nonspecific response to the cell injection itself, injection with mouse NIH3T3 cells did not increase the plasma C5a level in mice (Fig. 1D). To demonstrate C5a originating from SL4 cells has pro-metastatic effects, C5a was down-regulated by stable transfection of SL4 cells with plasmids expressing C5-shRNA, down-regulation of C5 in SL4 cells indeed reduced tumor metastasis ability (Fig. 2A). These results support a role for colon cancer cells in promoting C5a production.

Disruption of Host C5aR Signaling Inhibits Tumor Metastasis of Colon Cancer—C5a acts on specific receptors on various types of cells resulting in downstream immunomodulatory function. The receptor for C5a (C5aR) is normally expressed on myeloid cells, although its detection on non-myeloid cells has also been reported in the literature (33). We hypothesized that C5aR expression by phagocytes may contribute to the process of tumor development. To investigate whether the C5a-C5aR pathway is required for tumor metastases, we blocked C5a generation in tumor-bearing wild-type mice using an anti-mouse C5 monoclonal antibody (mAb; BB5.1) administered 1 day before the injection of tumor cells. An irrelevant IgG of the same isotype (MOPC) was used as a control (22). Anti-C5-treated mice had impaired hepatic metastases of colon cancer relative to mice treated with control IgG (Fig. 2B). These results support our hypothesis that C5a-C5aR signaling contributes to the development of hepatic metastasis by colon cancer cells.

We considered the possibility that the contribution of C5aR to the metastasis of colon cancer cells in our model could be explain by signaling either through C5aR on the injected SL4 cells or through C5aR on host cells. To distinguish these two possibilities and to further verify the role of C5a-C5aR signaling in colon cancer metastasis, we assessed tumor metastasis of SL4 cells injected into mice deficient in C5aR. Hepatic metastases from the C5aR-deficient mice had significantly reduced size as...
compared with the WT controls (Fig. 3A). To determine whether reduced formation of metastatic liver foci in C5a-R background is due to increased apoptosis, we examined apoptosis with TUNEL staining at early time points (day 1) after the same amount SL4 cell injection into spleen, there was no difference in apoptosis in WT and C5aR⁻/⁻ mice (Fig. 3C). These results verify that C5a signaling through C5aR is involved in colon cancer metastasis, and also clarify that C5aR expressed on host cells, rather than on the SL4 colon carcinoma cells, is involved.

C5a Promotes Metastasis by Inflammatory Cell Infiltration

In the tumor microenvironment, inflammatory cells and molecules influence almost every aspect of cancer progression, including the ability of the tumor cells to metastasize (15, 34). To assess the mechanism of C5a in hepatic metastases of colon cancer, we examined inflammatory cell infiltration in tumors of WT and C5aR⁻/⁻ mice. Inflammatory cell infiltration in collagenase-digested tumors was analyzed by flow cytometry. We excluded PI⁻ nonviable cells, gated on CD45⁺ population cells, and then compared the relative proportion of helper T lymphocytes (CD4⁺), cytotoxic T lymphocytes (CD8⁺), macrophages (F4/80⁺), neutrophils (Ly6G⁺), and dendritic cells (CD11c⁺) in liver metastatic foci from C5aR⁻/⁻ and WT mice. As shown in Fig. 4A, the proportion of infiltrating CD45⁺F4/80⁺ and Ly6G⁺ cells in liver metastatic tumors was significantly reduced in C5aR-deficient mice compared with WT mice. Similarly, the proportion of infiltrating CD45⁺Ly6G⁺ cells in liver metastatic tumors was significantly reduced in C5aR-deficient mice compared with WT mice. There were no significant differences in the amount of CD4⁺ or CD8⁺ T lymphocytes infiltrated into

FIGURE 3. Host C5a receptor deficiency inhibits tumor metastasis. A, gross hepatic metastases of colon cancer 14 days after intrasplenic injection of SL4 colon carcinoma cells (5×10⁵) into wild-type (WT) and C5aR-deficient mice (upper panel) (n = 5). H&E staining shows metastatic foci of liver colon cancer 14 days after intrasplenic injection of SL4 colon carcinoma cells (5×10⁵) into WT and C5aR⁻/⁻ mice (n = 5) (lower panel). The mean ± S.E. of tumor weights (upper panel) and tumor area % of liver tissue (lower panel) is quantified **, p < 0.01 versus WT. B, immunohistochemical analysis of Ki-67 expression after injection of SL4 colon carcinoma cells into WT and C5aR⁻/⁻ mice is shown (n = 6). C, TUNNEL staining shows apoptosis of liver colon cancer 1 day after intrasplenic injection of SL4 (5×10⁵) into WT and C5aR⁻/⁻ mice (n = 4).
tumors in C5aR-deficient mice compared with WT mice (data not shown). We also assessed the inflammatory cells in the blood of tumor-bearing mice and did not detect significant differences in the inflammatory cells in the blood (Fig. 4B). Thus, the inhibition of tumor metastasis in C5aR-deficient mice is specifically associated with reduced infiltration into the tumor of neutrophils, monocytes, and dendritic cells, all of which are of myeloid origin (35).

To verify that the infiltrating cells express C5aR, we performed double immunofluorescence staining with antibodies against C5aR, F4/80, and Ly6G (Fig. 5). Overlap was observed between C5aR and each of these myeloid markers, thus confirming that the infiltrating myeloid cells express C5aR.

**Bone Marrow-derived Cells Facilitate Tumor Metastasis in C5aR-deficient Mice**—Our results suggest that C5aR affects the infiltration of myeloid cells into metastatic foci in the liver. To determine whether bone marrow origin contribute to hepatic metastasis of colorectal cancer, we performed bone marrow (BM) transplantation and created C5aR-chimeric mice (23). Two months after BM transplantation, mice were inoculated with SL4 colon cancer cells. As shown as Fig. 5, the tumor size was dependent on the genotype of the cells that were transplanted. Transplantation of bone marrow from C5aR-deficient mice (KO(BM)) conferred a reduced size of the hepatic metastases of colorectal cancer as compared with bone marrow from WT mice (WT(BM)) for irradiated recipient mice of WT or C5aR(−/−) genotype (Fig. 6). Thus, these results verify that the inflammatory cells infiltrating into the tumor originate from the bone marrow, and that C5a facilitates their recruitment to the tumor site.

**Blockade of C5aR Reduces MCP-1 Release in Macrophage**—We postulated that the strong chemotaxtractant activity of C5a (36) may be derived, in part, from the activation of chemokines. MCP-1/CCL2 has potent monocyte chemotactic activity and is responsible for the recruitment of immunosuppressive macrophages that promote tumor growth (37, 38). Therefore, we determined whether C5a regulates inflammatory cell infiltration by promoting the expression of MCP-1 or other chemokines in hepatic metastatic foci of colon cancer. The expression of MCP-1 mRNA was markedly decreased in metastatic foci in C5aR-deficient mice, although there were no significant differences in the mRNA expression of other chemokines tested (Fig. 7A). To further support these findings, we assessed protein levels by CBA assay for macrophages and neutrophils isolated from mice. MCP-1 was highly expressed in macrophages. The amount of MCP-1 was not statistically different between WT and C5aR(−/−) mice; however, the addition of 50 nM C5a to the culture medium for 6 h caused a statistical increase in the levels of MCP-1, macrophage inflammatory proteins 1α (MIP-1α), and macrophage inflammatory proteins 1β (MIP-1β) in WT cells but not in C5aR(−/−) macrophages (Fig. 7B). To gain mechanistic insights into a functional link between C5a/C5aR-mediated signaling and inflammatory molecules, we examined C5a/C5aR-mediated inflammatory signaling, namely, mitogen-activated protein kinases (MAPK) and phosphoinositide

**FIGURE 4.** C5aR deficiency decreases inflammatory cell infiltration in hepatic metastases of SL4 colon cancer. A, CD4+ T lymphocytes (CD45+CD3+CD4+) and CD8+ T lymphocytes (CD45+CD3+CD8+) (left panel); and monocytes (CD45+CD11b+), neutrophils (CD45+Ly6G+), and dendritic cells (CD45+CD11C+) (right panel) were quantified by flow cytometry analyses of blood from WT and C5aR(−/−) mice. Data represent the mean ± S.E. for n = 5 mice per group. *p < 0.05 versus WT mice.

**FIGURE 5.** C5aR protein expression in tumor infiltrating macrophages and neutrophils. A, double-color immunofluorescence analyses of macrophages and C5aR expression in metastatic foci in the liver from WT and C5aR(−/−) mice. The sections were immunostained using a combination of anti-F4/80 and anti-C5aR antibodies. Bars = 25 μm. B, double-color immuno-fluorescence analyses of neutrophils and C5aR expression in metastatic foci in the liver from WT and C5aR(−/−) mice. The sections were immunostained using a combination of anti-Ly6G and anti-C5aR antibodies. Bars = 50 μm.
3-kinase (PI3K)-AKT (39), C5a/C5aR-activated AKT signal increased the expression of inflammatory molecules, which was blocked by Akt inhibition (MK-2206) (Fig. 7C). These results suggest that the levels of MCP-1 and other chemokines produced by macrophages may be dependent on C5a signaling through C5aR.

**C5aR Deficiency Enhances the Expression of Immune Stimulatory Genes and Reduces Metastases Ability**—To assess the effect of C5aR in the immune response to tumor metastasis; we evaluated the expression of several immune-related molecules in the tumor microenvironment. Total RNA was extracted from metastatic foci of colon cancer in the livers of C5aR-deficient and WT mice. Real-time PCR was performed for 8 immune-associated genes. The expression of pro-inflammatory nitric-oxide synthase 2 and IL-23 was significantly increased in the metastatic foci of colon cancer in the livers of C5aR-deficient mice (Fig. 8A). Furthermore, the expression of anti-inflammatory arginase 1, TGFβ, and IL-10 was significantly decreased in C5aR-deficient mice (Fig. 8B), whereas the expression of IL-1β and IL-12 p40 and p35 was essentially unchanged. To establish a causal role for C5a-stimulated chemokines from macrophage in the liver metastasis of colon cancer cells, we performed in vitro invasion assays using Matrigel-loaded transwell chambers. We found C5a-stimulated WT macrophage increased SL4 cell invasion in vitro, compared with non-stimulated WT macrophage, and importantly, C5a failed to cause SL4 cell invasion when C5aR was deficient in macrophage (Fig. 8C). These results are consistent with a role for C5a in the generation of an immunosuppressive tumor microenvironment.

**DISCUSSION**

Tumor metastasis is a complex event that requires interactions between tumor cells and the surrounding stroma (40). Inflammatory cells and molecules influence almost every aspect of this process (2, 41). Moreover, for colorectal cancer, the prognosis is closely associated with the presence of hepatic and other metastases (42). In this study, we have demonstrated the contribution of complement C5a to hepatic metastasis. We demonstrated that colon cancer cell lines can generate C5a and that C5a levels increase upon tumor progression. C5aR deficiency in a murine colon cancer model reduces hepatic metastasis, as well as inflammatory cell infiltration. Macrophage-derived MCP-1 and other cytokines are decreased in C5aR-deficient mice, suggesting a pathway for the associated levels of inflammatory cell infiltration. Furthermore, using bone mar-
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Our results showed that even though loss of C5aR significantly reduced liver metastasis but it does not completely abrogate liver metastasis, this suggest that the C5a-C5aR axis is necessary but not sufficient for liver metastasis, and other events such as senescence (46) and angiogenesis (47) are also required for liver metastasis.

These experiments collectively suggest that C5aR signaling promotes metastasis of SL4 tumors. The enhanced infiltration of inflammatory cells in C5aR-deficient mice suggests the possibility that C5a has immunomodulatory functions in tumor metastasis. Several studies with animal experimental models, as well as studies in humans, have demonstrated a crucial function for inflammatory cells in tumor immunity (24, 48, 49). Infiltration of leukocytes and macrophages are molecular signatures linked to a poor prognosis in cancer patients (50). Therefore, our findings that C5aR-deficient mice display reduced infiltration of macrophages and neutrophils in liver metastatic tumors suggest that C5aR deficiency can delay tumor metastasis by promoting immunosurveillance function in the tumor microenvironment. One of the important mechanisms used by malignant tumors to suppress the immune response to tumor antigens is abnormal myeloopoiesis, as well as the recruitment of myelomonocytic cells to the tumor site and peripheral lymphoid organs. In neoplasias, macrophages and neutrophils are recruited into the tumor from the peripheral circulation by chemokines. Our observation that MCP-1 expression is increased in metastatic foci of colon cancer in the liver provides a mechanism to explain the reduced infiltration and is consistent with previous work that demonstrates a direct correlation between the expression of MCP-1 and macrophage infiltration in human breast cancer tissues (37).

We have demonstrated an immunosuppressive capacity of C5a in our model. Immunosuppression by complement C5a has also been reported in a cervical cancer model (15), for which the generation of C5a in the tumor microenvironment enhances tumor growth by the recruitment of myeloid-derived suppressor cells and the suppression of the antitumor T cell-mediated response, including the expression of key immunosuppressive molecules within tumors (15). These results are in full agreement with our studies. We found that the expression of anti-inflammatory molecules arginase 1, IL-10, and TGF-β was down-regulated; whereas the expression of the key pro-inflammatory molecules nitric-oxide synthase 2 and IL-23 was up-regulated. Furthermore, we found that macrophage stimulation with C5a promote the invasion ability of SL4 colon cancer cells.

In conclusion, our results further support the role of complement C5a in tumor metastasis. Further studies are needed to extend our findings to other metastatic models of cancers. The findings reported here not only introduce a new complement-mediated mechanism of tumor-dependent immunosuppression, but also provide preliminary evidence of the potential utility of complement inhibition as a therapeutic option in anticancer therapy. Given that complement inhibition over-rides tumor-dependent immunosuppression, this therapeutic approach may also hold promise as a supplement to antitumor vaccines.
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