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Opposing Roles for Complement Component C5a in Tumor Progression and the Tumor Microenvironment

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Promoting complement (C) activation may enhance immunological mechanisms of anti-tumor Abs for tumor destruction. However, C activation components, such as C5a, trigger inflammation, which can promote tumor growth. We addressed the role of C5a on tumor growth by transfecting both human carcinoma and murine lymphoma with mouse C5a. In vitro growth kinetics of C5a, control vector, or parental cells revealed no significant differences. Tumor-bearing mice with C5a-transfected xenografted tumors had significantly less tumor burden as compared with control vector tumors. NK cells and macrophages infiltrated C5a-expressing tumors with significantly greater frequency, whereas vascular endothelial growth factor, arginase, and TNF-α production were significantly less. Tumor-bearing mice with high C5a-producing syngeneic lymphoma cells had significantly accelerated tumor progression with more Gr-1+CD11b+ myeloid cells in the spleen and overall increased CD4+ and CD8+ T cells in the tumor, tumor-draining lymph nodes, and the spleen. In contrast, tumor-bearing mice with low C5a-producing lymphoma cells had a significantly reduced tumor burden with increased IFN-γ-producing CD4+ and CD8+ T cells in the spleen and tumor-draining lymph nodes. These studies suggest concentration of local C5a within the tumor microenvironment is critical in determining its role in tumor progression. The Journal of Immunology, 2012, 189: 000–000.

Complement (C) is an important component of the immune system, and activation of the C cascade occurs via three pathways (1). The C cascade is highly regulated at several levels by C regulatory proteins, such as CD55 (2, 3). CD55 (decay-accelerating factor) is expressed on nearly all cells of the body and overexpressed on tumor cells. It is responsible for the accelerated dissolution of the C3 and C5 convertases and eliminates release of anaphylatoxins (2). C activation component C5a is a potent immune mediator, activating immune cells upon interaction with the C5a receptor (C5aR), resulting in oxidative bursts in neutrophils, phagocytosis augmentation, and enzymatic granule release, as well as vasodilation of local blood vessels to enhance immune cell entry and exchange of cells and factors from the circulation into the local tissue (4). C5a can be quickly degraded by C5a desArg by carboxypeptidases available abundantly in tissues and serum (5).

Translational research efforts support a positive role for C5a in anti-tumor mAb therapy. Yeast-derived β-glucan polysaccharide adjuvant therapy has been demonstrated to prime C receptor 3 (CR3) on neutrophils for enhanced tumor killing (6). C activation during β-glucan therapy is critical for efficacy, because it leads to the release of C5a responsible for recruiting β-glucan–primed neutrophils (7). Expression of CD55 by human ovarian carcinoma SKOV-3 inhibits the release of C5a, thus limiting therapeutic efficacy of combined β-glucan with anti-Her2/neu Ab treatment in vivo (8). In addition, in vitro studies involving human breast cancer cells demonstrated improvement in tumor cell killing by neutrophils following treatment of cells with anti-Her-2/neu mAb fused to either C5a or C5a desArg (9). Furthermore, murine mammary sarcoma EMT6 cells transfected with mouse C5a, which were capable of producing low levels of C5a and inducing minimal migration of J774 macrophage cells in vitro, had the most substantial reduction in tumor growth and regression as compared with control EMT6 cells in vivo (10). Contrary to these findings, a more recent study demonstrated a protumorigenic role of C5a generated via the classical pathway within the tumor microenvironment (11). In this TC-1 murine cervical cancer model, C5a was shown to recruit significantly more myeloid-derived suppressor cells (MDSCs) and to enhance the production of immunosuppressive reactive oxygen species and reactive nitrogen species by these cells resulting in the inhibition of the antitumor-specific CD8+ T cell response and increased tumor burden (11). Thus, the role of C5a in the tumor remains controversial (12–14).

In the current study, we addressed the role of C5a on tumor growth by transfecting tumor cells with mouse C5a. We found that the local C5a concentration within the tumor microenvironment is critical in determining its role in tumor progression.

Materials and Methods
C5a transfection and mouse tumor models
Murine lymphoma RMA and human ovarian carcinoma SKOV-3 cells from the American Type Culture Collection (Manassas, VA) were transfected with secreting murine C5a, or empty vector DNA plasmids provided by Dr.
F. Liang (Veterinary School of Medicine, Louisiana State University, Baton Rouge, LA). Transfection using the Lipofectamine reagent (Invitrogen, Carlsbad, CA) was performed, according to the manufacturer’s guidelines. Supernatants from wells of confluent G418-selected cells were screened by C5a ELISA (BD Biosciences, Bedford, MA), and chemotaxis assay was performed using murine macrophage J774 cells. Positively transfected cells, identified by ELISA, were plated in a limiting dilution assay to isolate single clones expressing C5a for injection and were then passaged in vivo at various following excision, were rescreened by ELISA and immunohistochemistry (IHC) for C5a expression. In vitro tumor cell growth rates were measured in real time, using the Acea system (Acea Biosciences, San Diego, CA) as described previously (15). Each cell line was plated in quadruplicate in the ACEA 16-well plate. The cell index was recorded to reveal tumor cell growth.

All mice used in vivo studies were approved and treated following requirements of the Institutional Animal Care and Use Committee of the University of Louisville. Studies were performed on both SCID mice 6-10 wk of age purchased from Taconic (Germantown, NY) or Harlan Laboratories (Fox Chase Cancer Center, Philadelphia, PA) and wild-type (WT) C57BL/6 mice (National Cancer Institute, Frederick, MD). For the SKOV-3 tumor model, 7–10 × 10^5 C5a-transfected or control vector (CV)-transfected SKOV-3 cells were injected s.c. with Matrigel (BD Biosciences). For the murine lymphoma RMA model, female C57BL/6 mice (National Cancer Institute, Frederick, MD) were developed with tetramethylbenzidine conductivity substrate (BioFX Sciences, San Diego, CA) was added at 2 mg/ml. Slides were incubated in the humidity chamber overnight at 4˚C. Streptavidin–HRP (Vector Laboratories) was prepared and applied to slides. Slides were rinsed and then incubated with the 3-amino-9-ethylcarbazole substrate solution (BioFX Laboratories, Owings Mills, MD), and the OD value was measured at 450 nm.

**IHC and immunofluorescent staining**

Frozen tumor tissue sections were fixed in ice-cold acetone. Appropriate blocking steps were performed on tissue samples including 3% hydrogen peroxide, 3% BSA in 1× PBS, and avidin/biotin blocking kit (Vector Laboratories Burlingame, CA). Biotinylated rat anti-mouse C5a Ab (BD Biosciences) was applied at a concentration of 2 μg/ml. Assays were developed with tetramethylbenzidine conductivity substrate (BioFX Laboratories). Slides were counterstained with hematoxylin. Images were acquired with Aperio ScanScope digital scanners (Aperio, Vista, CA). For immunofluorescent (IF) staining, slides were stained with DX5 Alexa Fluor 647 or F4/80 allopochycin (BioLegend, San Diego, CA) with appropriate isotype controls. Slides were washed, and DAPI (Invitrogen) nuclear stain was added. Images were acquired by Leica TCS SPS confocal microscopy system (Leica Microsystems, Buffalo Grove, IL).

**In vitro cytotoxicity**

In vitro cytotoxicity assay was performed using the Acea system as described previously (15). Several innate immune cell populations were used in various cytotoxicity experiments. Single-cell suspensions prepared from naive SCID mouse spleens were added to a flask and cultured briefly for 1 d. Macrophages were cultured with varying concentrations of C5a (R&D Systems) for 24 h and then cocultured with CD4 T cells (2:1 ratio) in the presence of OVA (15 μg/ml) for an additional 3 d. For regulatory T cell (Treg) induction assay, macrophages were cocultured with CD4 T cells in the presence of OVA and varying amounts of C5a for 4 d. Cells were restimulated with PMA/ionomycin in the presence of GolgiPlug for 4 h. Intracellular IFN-γ, IL-17, or Foxp3 staining was performed.

**Graphing and statistical analysis**

Prism 5.0 (GraphPad Software, San Diego, CA) was used in creating graphs and analyzing data collected from in vitro and in vivo studies. Following C5a ELISA, the standard curve was graphed, a linear regression test was performed, and sample concentrations were extrapolated from the standard curve. Analysis of tumor growth significance and significance between the protocols were calculated as 2−ΔΔCt, where ΔCt = Ct_target gene – Ct_GAPDH and ΔΔCt = ΔCt_induced − ΔCt_REFERENCE.

**Cytospin and stain**

Sorted cells were applied to the Shandon premade cuvettes (Shandon, Pittsburgh, PA) and slide and spun in Shandon Cytospin 3 centrifuge. After spinning, cells were fixed to the slide with methanol and stained with Propidium iodide (Molecular Probes, Eugene, OR). DNA content was evaluated by flow cytometry.

**Results**

C5a-expressing tumor cells have significantly reduced growth in SCID mice

The human ovarian adenocarcinoma cell line, SKOV-3, has been shown to overexpress the C regulatory protein CD55 (8). CD55 accelerated inhibition of C5a release and has been demonstrated to result in diminished tumor infiltration of β-glucan–primed neutrophils (8). To determine whether C5a expression could enhance the recruitment of innate leukocytes to the tumor microenvironment, eliminating the negative effects of CD55, SKOV-3 cells were transfected with mouse C5a or CV. SKOV-3 WT cells were confirmed not to express C5a by ELISA and IHC, whereas SKOV-3 C5a tumors had abundant C5a deposition (Supplemental Fig. 1A). In vitro chemotaxis assay indicated supernatants from SKOV-3 C5a tumor cells enhanced migration of J774 cells (data not shown), suggesting that C5a secreted from transfected tumor cells was functionally active.

To determine whether the transfection of C5a induced any growth disparities on cells, in vitro growth kinetics was monitored.

**Quantitative real-time PCR**

Small portions of excised tumors were removed with a scalpel blade and weighed. Tumor tissues with similar weight were placed in 1 ml TRIzol reagent (Invitrogen) and kept at −80°C. Sorted cells were also placed in TRIzol. RNAs were isolated using a Qiagen RNeasy kit, according to the manufacturer’s instructions (Qiagen, Valencia, CA). A set amount of RNA was also used to control for differences in tumor size. After reverse transcription into cDNA with a Reverse Transcription kit (Bio-Rad, Hercules, CA), quantitative real-time PCR (qRT-PCR) was then performed on Bio-Rad MyIQ single-color RT-PCR detection system using SYBR Green Supermix (Bio-Rad), and gene-specific primers were summarized in the Supplemental Table I. Gene expression levels were normalized to GAPDH housekeeping gene, and data were represented as fold differences by the 2−ΔΔCt method, where ΔCt = Ct_target gene − Ct_GAPDH and ΔΔCt = ΔCt_induced − ΔCt_REFERENCE.
Both C5a- and CV-transfected clones displayed an initial in vitro growth enhancement over SKOV-3 WT cells but was no longer apparent after 12 h of culture; all three cell lines were determined to grow equally well (Supplemental Fig. 1B). Selected clones were then used to observe their in vivo tumor growth. Using the SCID-immunocompromised/SKOV-3 tumor model was beneficial on two fronts. It permitted for focus on the effect of C5a on innate leukocyte infiltration and functional activity of these cells in the tumor, exclusively, which were hypothesized to be the main targets. In addition, the model allowed for study of an aggressive human carcinoma that overexpresses CD55 (8), resulting in the inhibition of C activation at the C3 and C5 convertase step, eliminating local C5a release. All tumor cell lines demonstrated similar initial in vivo growth; however, beginning around day 24 postinjection, SKOV-3 C5a tumors revealed significant reduction in tumor progression (Fig. 1A). Upon excision at an endpoint time between 31 and 38 d for three separate experiments, C5a-expressing tumors weighed significantly less than both CV and WT tumors (Fig. 1B).

Enhanced infiltration of innate immune cell subsets in SKOV-3 C5a-expressing tumors

Innate immune cells have been shown to be important to mount an antitumor response (7, 16, 17) as well as play an important role in sustaining the immunosuppressive environment and angiogenic switch promoting tumor growth and metastasis (18, 19). Expression of C5a from the tumor environment may harness the antitumor response of these cells. We found a slight increase in circulating DX5+ NK cells from spleen and peripheral blood samples and a surprising decrease in splenic Gr-1+CD11b+ cells compared with naive SCID (data not shown). Tumors were then examined for the role of C5a in enhancing the migration of innate leukocytes into the tumor tissue. Flow cytometric analysis revealed that there was no difference in the percentage of Gr-1+CD11b+ cells infiltrating the tumor (Fig. 2A). However, C5a tumors showed an increased percentage of infiltrating DX5+CD11b+ NK cells (Fig. 2B) and F4/80+CD11b+ subsets of macrophages (Fig. 2C). Taken together, C5a appears to be enhancing the infiltration of NK cells and macrophages in tumor, two distinct subsets of innate immune cells that have been shown to be important in tumor immunity.

Tumor microenvironment analysis reveals a significant decrease in the production of protumorigenic factors

Next, we examined pro- and antitumorigenic gene levels to identify alterations in the tumor microenvironment as a result of C5a expression. When the total tumor samples were analyzed by qRT-PCR, many genes evaluated did not show a difference: inducible NO synthase (iNOS), TGF-β, IL-6, IL-10, IL-12, IFN-γ, granzyme B, or perforin (Fig. 3A; data not shown). However, the mRNA levels of vascular endothelial growth factor (VEGF), arginase, and TNF-α were significantly lower in C5a-expressing tumors (Fig. 3A). To further delineate the source of VEGF and iNOS influenced by C5a, both CD11b+ leukocytes and CD11b− tumor cells were analyzed (Fig. 3B). These data indicated the activity of C5a resulted in reduction of VEGF from tumor cells. In contrast, iNOS mRNA level was significantly lower in C5a-expressing SKOV-3 tumor-infiltrating leukocytes (Fig. 3B). Isolation of F4/80+ infiltrates followed by qRT-PCR demonstrated no difference in the case of VEGF, iNOS, TNF-α, or IL-12 (data not shown); however, F4/80+ cells infiltrating SKOV-3 C5a tumors made significantly less arginase (Fig. 3C), suggesting an antimacrophage phenotype.

The presence of C5a renders naive innate leukocytes more cytotoxic to tumor cells and decreases Gr-1+CD11b+ cell inhibitory activity

In vitro studies were enlisted to determine whether C5a could enhance the cytotoxicity of tumor cells by naive innate leukocytes. Compared with SKOV-3 CV cells, SKOV-3 C5a cells were killed at a significantly higher percentage by the naive leukocytes (Fig. 4A). In addition, NK cells from naïve mice had significantly higher killing activity for SKOV-3 C5a tumor cells as compared with SKOV-3 CV cells (Fig. 4B). These results indicate C5a has activating potential of naive neutrophils and/or NK cells for superior effector function.

As shown in Fig. 2A, the frequency of Gr-1+CD11b+ cells was not significantly altered in a C5a-expressing tumor. Next, we examined the inhibitory activity of tumor-infiltrating Gr-1+CD11b+ cells on naive, nonadherent leukocytes-mediated cytotoxicity of tumor cells. Nonadherent leukocytes from naive SCID mice showed significant level of cytotoxicity against SKOV-3 tumor cells. The addition of Gr-1+CD11b+ cells from either SKOV-3 C5a or SKOV-3 CV tumors significantly inhibited cytotoxic activity (Fig. 4C). However, Gr-1−CD11b+ cells from SKOV-3 C5a tumors were significantly less suppressive. In the absence of the naive leukocytes, neither tumor-isolated Gr-1−CD11b+ cells led to SKOV-3 WT tumor cell destruction. They actually promoted tumor cell growth in vitro. The images of the Gr-1−CD11b+ cells demonstrated nearly all these cells were morphologically similar to neutrophils (Fig. 4D).
C5a-expressing tumor cells have significantly accelerated growth in immunocompetent mice

Although the SCID mouse model allows us to study C5a effect on innate immune cells, these mice lack adaptive T/B cells. To determine C5a-expressing tumor growth in immunocompetent host, murine lymphoma RMA cells with or without C5a expression were implanted in WT C57BL/6 mice. Surprisingly, C5a-expressing tumors (RMA-3CF4) grew significantly faster than CV-transfected cells (RMA-CVA1) from all three independent experiments (Fig. 5A; data not shown). The frequency of Gr-1+ CD11b+ MDSCs was significantly higher in RMA-3CF4 spleen compared with RMA-CVA1 although no difference was observed in TDLN and tumors (Fig. 5B). Innate immune cells including F4/80+ macrophages and NK1.1+ NK cells were largely unchanged (Fig. 5B).

Strikingly, the frequency of both CD4+ and CD8+ T cells from the spleen, TDLN, and tumor was significantly lower in C5a-expressing tumor-bearing mice as compared with CV A1 controls (Fig. 5C, 5D). However, significantly more of the RMA-3CF4 spleen- and TDLN-infiltrating CD8+ T cells produced IFN-γ, although a similar percentage of IFN-γ-producing CD8+ T cells was observed in the tumor. In addition, the percentage of CD4+ T cells from RMA-3CF4 spleen and tumor-producing IFN-γ displayed a slightly different pattern. Significantly more of the splenic CD4+ T cells produced IFN-γ; however, significantly less tumor-infiltrating CD4+ T cells produced IFN-γ when compared with CV A1 controls. In addition, splenic Tregs were significantly increased in C5a-expressing tumor-bearing mice (Supplemental Fig. 2A) as compared with CVA1 animals. Taken together, RMA-3CF4 tumor-bearing mice had overall significantly lower percentages of infiltrating CD4+ and CD8+ T cells in the spleen, TDLN, and tumor, with an increased percentage of these subsets producing IFN-γ in the spleen and TDLN by CD8+ T cells and in the spleen by CD4+ T cells but decreased IFN-γ production by tumor-infiltrating CD4+ T cells.

Low C5a production from tumor cells significantly decreases tumor growth

Given the contradictory data generated from the different models, we noted C5a concentrations detected from SKOV-3 versus RMA cells differed. RMA-3CF4 cells secreted higher levels of C5a as compared with CV A1 animals. Taken together, RMA-3CF4 tumor-bearing mice had overall significantly lower percentages of infiltrating CD4+ and CD8+ T cells in the spleen, TDLN, and tumor, with an increased percentage of these subsets producing IFN-γ in the spleen and TDLN by CD8+ T cells and in the spleen by CD4+ T cells but decreased IFN-γ production by tumor-infiltrating CD4+ T cells.
terns also differed between the groups of RMA tumors. Percentages of infiltrating CD4+ and CD8+ T cells were significantly increased in the spleen but significantly decreased in TDLN in RMA-1474 tumor-bearing mice. In addition, splenic Treg were comparable in RMA-1474 versus CV A1 tumor-bearing mice (Supplemental Fig. 2B). Although there were significantly fewer of the T cell subsets infiltrating RMA-3CF4 tumors with high levels of C5a, no difference was observed in the percentage of infiltration of tumors between CV A1 and RMA-1474 with low C5a (Fig. 6D, 6E). Similar to RMA-3CF4 T cell populations, a significantly greater percentage of CD4+ and CD8+ T cells produced IFN-γ in the spleen and TDLN of RMA-1474 tumor-bearing mice.

C5a drives Th1 and Treg differentiation via concentration-dependent manner

C5a has been previously demonstrated to stimulate Th17 cell differentiation and trigger autoimmune arthritis and experimental autoimmune encephalomyelitis (20, 21). We next examined whether Th cell differentiation mediated by C5a was also concentration dependent. To this end, naive OVA TCR transgenic CD4 T cells were cultured with macrophages in the presence of varying levels of C5a. As shown in Fig. 7A, C5a at the concentrations of 100 and 300 ng/ml significantly promoted Th1 cell differentiation as revealed by more IFN-γ production. However,

FIGURE 3. The altered tumor microenvironment by C5a. (A) Total tumor samples were collected, and RNAs were extracted. qRT-PCR data revealed the downregulation of VEGF, arginase, and TNF-α mRNA levels in C5a-expressing tumors. *p < 0.05, **p < 0.01. (B) Sorted CD11b+ innate immune cells and CD11b− tumor cells were performed for qRT-PCR analysis. Data indicate that VEGF mRNA level is significantly decreased in C5a-expressing tumor cells, whereas the CD11b+ cells sorted from SKOV-3 C5a have significantly lower levels of iNOS mRNA. *p < 0.05. (C) The SKOV-3 C5a-infiltrating F4/80+ macrophages expressed significantly lower levels of arginase mRNA. *p < 0.05.
C5a at the higher level (500 ng/ml) significantly decreased Th1 differentiation (Fig. 7A). In contrast, increasing concentrations of C5a gradually decreased Treg induction (Fig. 7C). C5a at 100 and 300 ng/ml significantly decreased Treg induction, whereas C5a at the higher concentration significantly promoted Treg induction (Fig. 7C). These effects were completely abrogated in C5aR-deficient mice (data not shown). No difference was observed for Th17 cell differentiation (Fig. 7B). These data suggest that C5a-mediated Th1 and Treg differentiation appear to be bell shaped.

Discussion

Thus far, the role of C5a in the tumor microenvironment has been inconclusive, with previously published studies demonstrating either C5a release from the tumor resulted in reduced tumor growth (10) or C5a-enhanced immune suppressive cells and supported tumor growth (11). We have demonstrated in this study the SKOV-3 xenograft model support of a proimmunogenic, antitumor role for C5a released in the tumor microenvironment. C5a is acting on host immune cells and indirectly on tumor cells to alter the cytokine milieu and enhance tumor infiltration and cytotoxic function of innate immune effector cells. The C5a effect in the immunocompetent model appears to be concentration dependent. High C5a levels stimulate tumor growth with significantly decreased infiltration of CD4+ and CD8+ T cells, whereas low levels of C5a within the tumor microenvironment decrease tumor progression. Therefore, local C5a concentration is critical in determining its role in tumor progression.

In the SKOV-3 xenograft model, the effect of C5a release in the tumor elicits minimal changes in the periphery, and dramatic changes occur in the tumor microenvironment. Because SKOV-3 tumor cells lack C5aR expression, the reduced in vivo growth of SKOV-3 tumor cells expressing C5a is likely due to the responses by host innate immune cells. As demonstrated by the in vitro cytotoxicity assay, the C5a secreted from tumor cells enhances the effector functions of neutrophils and NK cells in vitro and renders them more cytotoxic to the tumor cells. Similarly, a recent study showed that C5a–C5aR interaction enhanced NK cell IFN-γ production in sepsis (22). C5a release in the tumor also enhances the recruitment of innate immune cells to the tumor. Enhanced recruitment of the DX5+CD11b+ NK cells into the tumor is beneficial because of the tumor cytotoxic and immune enhancing potential of NK cells (23, 24). In solid tumors, NK cell penetration is noted as a positive prognostic factor, but most solid tumors demonstrate inferior NK cell infiltration (23). In a C5a-expressing tumor, macrophages are also significantly increased. Macrophages play a dominant role in influencing other immune cells and tumor growth depending on phenotype (25, 26). Two extreme ends of macrophage polarization have been characterized, based on the stimulatory factors and products released by the cells: M1 (anti-tumorigenic) and M2 (protumorigenic) macrophages (25, 27). As shown in the current study, macrophages from C5a-expressing tumor have significantly low mRNA levels of arginase, suggesting an M1 phenotype. Although the frequency of Gr-1+CD11b+ cells is similar in SKOV3 C5a and SKOV-3 CV tumor, the abundant expression of C5aR on these cells renders them most sensitive to local C5a concentrations. Indeed, Gr-1+CD11b+ cells from SKOV-3 C5a tumor have less immune-suppressive effect as compared with those from SKOV-3 CV tumor. Thus, local C5a may promote innate immune cell traffic into tumor, and once immune cells are recruited, C5a can activate and enhance the cytotoxic functions.
FIGURE 5. C5a-expressing lymphoma cells have significantly enhanced tumor progression. (A) WT C57BL/6 mice were injected with C5a-expressing lymphoma RMA-3CF4 cells or RMA CVA1 cells \((n = 10)\), and tumor growth was recorded. Data are shown as mean ± SEM. ** p < 0.001. (B) The spleen, TDLN, and tumor from tumor-bearing mice were prepared for single-cell suspensions. Cells were then stained with Gr-1, CD11b, F4/80, or NK1.1. Representative dot plots and summarized data are shown. (C) Cells were stimulated with PMA/ionomycin and surface stained with CD8 and IFN-γ intracellularly. Representative dot plots (cells were gated on the CD8+ T cells), summarized IFN-γ-producing CD8+ T cells, and total CD8+ T cells are shown. (D) Cells were stimulated with PMA/ionomycin and surface stained with CD4 and IFN-γ intracellularly. Representative dot plots (cells were gated on the CD4+ T cells), summarized IFN-γ-producing CD4+ T cells, and total CD4+ T cells are shown.
FIGURE 6. Low C5a-expressing lymphoma cells have significantly decreased tumor progression. (A) C5a levels of RMA cells transfected with C5a or CV. Data indicate that the RMA 3CF4 clone secreted higher levels of C5a than the RMA-1474 clone. (B) WT C57BL/6 mice were injected with low C5a-expressing lymphoma RMA-1474 cells (n = 10) or RMA CVA1 cells (n = 15), and tumor growth was recorded. Data are shown as mean ± SEM. *p < 0.05. (C) Cells from spleen and tumor from tumor-bearing mice were stained with Gr-1 and CD11b. Representative dot plots and summarized data are shown. (D) Cells were stimulated with PMA/ionomycin and surface stained with CD8 and IFN-γ intracellularly. Representative dot plots (cells were gated on the CD8+ T cells), summarized IFN-γ-producing CD8+ T cells, and total CD8+ T cells are shown. (E) Cells were stimulated with PMA/ionomycin and surface stained with CD4 and IFN-γ intracellularly. Representative dot plots (cells were gated on the CD4+ T cells), summarized IFN-γ-producing CD4+ T cells, and total CD4+ T cells are shown.
C5a also leads to events that can alter a local tumor environment. Significant changes in four important tumor and immune-regulating factors exist between SKOV-3 C5a and SKOV-3 CV tumors: arginase, iNOS, VEGF, and TNF-α. Arginase and iNOS are essential for MDSC-mediated immune-suppressive effect (28, 29). VEGF and its role in angiogenesis and tumor neovascularization are exploited by the tumor (30-32). We show that it is the tumor cells and not the immune-infiltrating CD11b+ cells that express significantly more VEGF. Because of the lack of C5aR expression on tumor cells, the mechanism of C5a is indirect. A recent study also demonstrated that C5a negatively regulates neovascularization and angiogenesis via secretion of soluble VEGF receptor 1 (33).

On the contrary, C5a-expressing tumor cells in immunocompetent mice showed more complex results. High C5a release in the tumor microenvironment promotes tumor progression, which is similar as TC-1 tumor model (11). The most striking finding from this model is the overall decreased frequencies of CD4+ and CD8+ T cells in the spleen, TDLN, and tumor in high C5a-expressing tumor-bearing mice. Although MDSCs significantly accumulated in the spleen, MDSCs have been shown to downregulate L-selectin expression on CD4+ and CD8+ T cells, thus decreasing their homing to sites where they should be activated (34, 35). The decreased CD4+ and CD8+ T cells in high C5a-expressing tumor-bearing mice could be due to the accumulated MDSCs in spleen. This is also supported by the data generated from low C5a-expressing tumors where MDSCs are not significantly altered, and CD4+ and CD8+ T cells are increased in spleen and decreased in TDLN but equivalent in tumor. The C system has recently demonstrated to be critical in regulating adaptive T cell responses (36). C activation can regulate CD4+ Th1, Treg, and Th17 cell differentiation (20, 21, 37–40) as well as CD8+ T cell immunity (41, 42). Indeed, IFN-γ-producing CD8+ T cells are significantly increased in the spleen and TDLN in both high and low C5a-expressing tumor-bearing mice, suggesting C5a can augment IFN-γ production by CD8+ T cells at distant sites. However, IFN-γ–producing CD4+ T cells are differentially regulated by C5a because tumor-infiltrating IFN-γ–producing CD4+ T cells are significantly decreased in high C5a-expressing tumors, whereas no difference is observed in C5a low-expressing tumor. This is further supported by the in vitro CD4+ T cell differentiation experiments showing C5a at 100 and 300 ng/ml promotes Th1 responses, whereas C5a at the higher concentration (500 ng/ml) inhibits Th1 and promotes Treg differentiation. In addition, splenic Treg are significantly increased in high C5a-expressing tumor-bearing mice while percentages of splenic Tregs are comparable in C5a low tumor-bearing animals as compared with CV tumor-bearing mice.

We thus hypothesize that tumor growth outcomes may differ tremendously because of C5a concentration levels in the local tumor microenvironment. High C5a release in the tumor microenvironment promotes tumor progression, which is similar as TC-1 tumor model (11). The most striking finding from this model is the overall decreased frequencies of CD4+ and CD8+ T cells in the spleen, TDLN, and tumor in high C5a-expressing tumor-bearing mice. Although MDSCs significantly accumulated in the spleen, MDSCs have been shown to downregulate L-selectin expression on CD4+ and CD8+ T cells, thus decreasing their homing to sites where they should be activated (34, 35). The decreased CD4+ and CD8+ T cells in high C5a-expressing tumor-bearing mice could be due to the accumulated MDSCs in spleen. This is also supported by the data generated from low C5a-expressing tumors where MDSCs are not significantly altered, and CD4+ and CD8+ T cells are increased in spleen and decreased in TDLN but equivalent in tumor. The C system has recently demonstrated to be critical in regulating adaptive T cell responses (36). C activation can regulate CD4+ Th1, Treg, and Th17 cell differentiation (20, 21, 37–40) as well as CD8+ T cell immunity (41, 42). Indeed, IFN-γ–producing CD8+ T cells are significantly increased in the spleen and TDLN in both high and low C5a-expressing tumor-bearing mice, suggesting C5a can augment IFN-γ production by CD8+ T cells at distant sites. However, IFN-γ–producing CD4+ T cells are differentially regulated by C5a because tumor-infiltrating IFN-γ–producing CD4+ T cells are significantly decreased in high C5a-expressing tumors, whereas no difference is observed in C5a low-expressing tumor. This is further supported by the in vitro CD4+ T cell differentiation experiments showing C5a at 100 and 300 ng/ml promotes Th1 responses, whereas C5a at the higher concentration (500 ng/ml) inhibits Th1 and promotes Treg differentiation. In addition, splenic Treg are significantly increased in high C5a-expressing tumor-bearing mice while percentages of splenic Tregs are comparable in C5a low tumor-bearing animals as compared with CV tumor-bearing mice.

We thus hypothesize that tumor growth outcomes may differ tremendously because of C5a concentration levels in the local tumor microenvironment. High C5a levels may lead to either overactivation of infiltrating cells or enhancement of an inflammatory setting to perpetuate tumor growth, angiogenesis, and suppression of the antitumor T cell infiltration. This may be analogous to sepsis, during which an overactivated C system (e.g., the release of high levels of C5a) disables innate immune cells, decreasing phagocytic function and resulting in an overall immunosuppressive state (43). Conversely, low levels of C5a may enhance infiltration of immune cells, and upon entry into the environment, C5a at low levels stimulates a more powerful antitumor immune response. However, quantitation of the chemoattractant C5a and its quick degradation by enzymes in the environment complicate pinpointing the critical concentration threshold of C5a. This is particularly important in the setting of in vivo anti-tumor mAb therapy. In addition, the C5a levels could be very different in the current model system where tumors continuously secrete C5a, whereas under natural conditions, C5a is
primarily produced through local complement fixation. More work needs to be done to determine whether the current findings are relevant for an in vivo immunotherapeutic setting. In addition, the differences observed in the two model systems need to be reconciled because of different tumor types, mouse strains, and the presence or absence of the adaptive immune system. Nevertheless, the evidence generated from previous studies (10, 11) and the current work support the hypothesis that C5a concentration holds the key in determining the response generated in the tumor.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary:

Table I. Primers for real-time PCR

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<th>Genes</th>
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Figure S1. C5a expression by transfected SKOV-3 cells and *in vitro*. (A) C5a levels of SKOV-3 cells by ELISA from SKOV-3 wildtype (WT), SKOV-3 C5a or SKOV-3 CV cells. Staining of tumor tissue sections following *in vivo* passage by IHC revealed expression of C5a (brown) in SKOV-3 C5a tumor but not in SKOV-3 WT tumor. Magnification: 20x. (B) *In vitro* SKOV-3 cell growth at different timepoints as indicated.
Figure S2. Splenic Treg infiltration in tumor-bearing mice. (A) Spleens from C5a-high expressing lymphoma RMA-3CF4 or RMA CVA1 were stained for CD4 and Foxp3. Representative dot plots (cells were gated on the CD4+ T cells) and summarized data are shown. (B) Spleens from C5a-low expressing lymphoma RMA-1474 or RMA CVA1 were stained for CD4 and Foxp3. Representative dot plots (cells were gated on the CD4+ T cells) and summarized data are shown.