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

Lacey Gunn, Chuanlin Ding, Min Liu, Yunfeng Ma, Chunjian Qi, Yihua Cai, Xiaoling Hu, Deep Aggarwal, Huang-ge Zhang and Jun Yan

*Published online 22 August 2012
http://www.jimmunol.org/content/early/2012/08/22/jimmunol.1200846*

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/08/22/jimmunol.1200846.DC1

Why The JI?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Opposing Roles for Complement Component C5a in Tumor Progression and the Tumor Microenvironment

Lacey Gunn,*†‡1 Chuanlin Ding,*† Min Liu,* Yunfeng Ma,* Chunjian Qi,*†‡ Yihua Cai,* Xiaoling Hu,* Deep Aggarwal,* Huang-ge Zhang,*†‡ and Jun Yan*†‡

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 decreased 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 vasodilatation 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 to C5a desArg by carboxypeptidases available abundantly in tissues and serum (5).

Received for publication March 16, 2012. Accepted for publication July 22, 2012.

This work was supported by research funding from National Institutes of Health Grants R01 CA86412 and R01 CA150947 and the Kentucky Lung Cancer Research Board (to J.Y.).

Address correspondence and reprint requests to Dr. Jun Yan, Tumor Immunobiology Program, James Graham Brown Cancer Center, Clinical and Translational Research Building, Room 319, University of Louisville, 505 South Hancock Street, Louisville, KY 40202. E-mail address: jun.yan@louisville.edu

*Division of Hematology/Oncology, Department of Medicine, James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202; †Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY 40202; and ‡Department of Oncology, Affiliated Hospital of Nanjing Medical University, Changzhou No. 2 People’s Hospital, Changzhou 213003, China

†L.G. and C.D. contributed equally to this work.

The online version of this article contains supplemental material.

Abbreviations used in this article: C, complement; C5AR, C5a receptor; CR3, C receptor 3; CV, control vector; IF, immunofluorescent; IHC, immunohistochemistry; iNOS, inducible NO synthase; MDSC, myeloid-derived suppressor cell; qRT-PCR, quantitative real-time PCR; TDLN, tumor-draining lymph node; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200846
ROLE OF C5a IN TUMOR PROGRESSION

PMA/ionomycin in the presence of GolgiPlug for 4 h. The staining was formed on ice. For intracellular staining, the cells were stimulated with measurement.

calculated as described previously (15).

mouse spleens using the EasySep magnetic beads kit (StemCell Technologies). For the murine lymphoma RMA model, female C57BL/6 mice transfected SKOV-3 cells were injected s.c. with Matrigel (BD Biosciences). For the SKOV-3 tumor model, 7–10 × 10⁶ 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 were implanted s.c. with RMA-C5a (3CF4), RMA-C5a (1474), or RMA-CVA1 cells (2 × 10⁶/mouse). Tumor growth was monitored by caliper measurement.

C5a ELISA

ELISA plates were coated with 2 µg/ml purified rat anti-mouse C5a mAb (BD Biosciences) for overnight at 4 °C. Culture supernatants from transfected cells and the biotinylated rat anti-mouse C5a detection Ab (BD Biosciences) was applied at a concentration of 2 µg/ml. Assays were developed with tetramethylbenzidine conductivity substrate (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 1X PBS, and avidin/biotin blocking kit (Vector Laboratories Burlingame, CA). Biotinylated rat anti-mouse C5a Ab (BD Biosciences) was added at 2 µg/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.

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 vitro and, 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 cell counting per 4 h. Intracellular IFN-γ, IL-17, or Foxp3 staining was performed.

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 Protocol Hema 3 solution I and II (Fisher Diagnostics, Middletown, VA). Cells were analyzed by microscopy under ×20 and ×40 magnification. Images were captured using the Nikon Eclipse E400.

T cell differentiation assay

Naive CD4 T cells from OT-II OVA TCR transgenic mice (Taconic) were purified by microbead separation (AutoMACS; Miltenyi Biotec). Macrophages were harvested from peritoneal cavity and purified with F4/80 microbeads. 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 concentrations of infiltrating cells or cytokine-secreting cells and qRT-PCR data analysis used the Student t test or two-way ANOVA.

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 introduced any growth disparities on cells, in vitro growth kinetics was monitored.
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. 1A). 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 antitumor macrophage 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 naive 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 promote 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).

**FIGURE 1.** In vivo growth of SKOV-3 tumor cells in SCID mice. (A) In vivo growth of SKOV-3 C5a and controls revealed a significant reduction in tumor growth of SKOV-3 C5a. Following s.c. injection of SCID mice with SKOV-3 tumor cell lines (n = 20, 16, and 8 for SKOV-3 C5a, CV, and WT, respectively), tumor growth was monitored by measuring two perpendicular diameters every 2–4 d. *p < 0.05, **p < 0.01, ***p < 0.001. (B) Tumor weight measurement when all animals were sacrificed. **p < 0.01, ***p < 0.001.
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 tumor-bearing mice (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 CVA1 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 compared with SKOV-3 C5a cells (data not shown). Next, we examined whether C5a levels from tumor cells have any impact on the tumor growth. As shown in Fig. 6A, RMA-1474 cells secreted low level of C5a, which is comparable to SKOV-3 C5a cells (data not shown). In contrast to RMA-3CF4 tumor, Gr-1+CD11b+ MDSC frequency was not significantly changed between RMA-CVA1 and RMA-1474 tumor-bearing mice (Fig. 6C). T cell infiltration pat-
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 4.** C5a promotes cytotoxicity of SKOV-3 tumor cells, whereas Gr-1+CD11b+ cells from SKOV-3 C5a tumors are significantly less immunosuppressive. (A) SKOV-3 C5a and CV tumor cells were cultured overnight, and the following day, nonadherent leukocytes from naive SCID mice as effector cells were added at a ratio of 20:1 (E:T). After 16 h of coculture, the percentage of cytotoxicity was calculated (n = 6). Data indicate that effector cells kill significantly more SKOV-3 C5a cells than SKOV-3 CV cells. **p < 0.01.** Similarly, purified NK cells from naive SCID mice were added to SKOV-3 C5a or CV tumor cells in vitro, and the percentage of cytotoxicity was determined following 24 h coculture. *p < 0.05. (B) SKOV-3 tumor cells were cocultured with nonadherent leukocytes as effector cells (20:1) in the presence or absence of Gr-1+CD11b+ cells sorted from CV- or C5a-transfected tumor (1:1) or without effectors but with sorted Gr-1+CD11b+ cells from tumor. The innate leukocytes demonstrated effective cytotoxicity of SKOV-3 tumor cells and cytotoxicity was significantly decreased in the presence of Gr-1+CD11b+ cells sorted from tumors. However, Gr-1+CD11b+ cells from SKOV-3 C5a tumors were significantly less suppressive. **p < 0.01. (D) Cytospin and stain of the Gr-1+CD11b+ cells sorted from the SKOV-3 tumors. Images were acquired at ×20 (left) and ×40 (right) magnification.
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.
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