CXCL12/CXCR4 Blockade by Oncolytic Virotherapy Inhibits Ovarian Cancer Growth by Decreasing Immunosuppression and Targeting Cancer-Initiating Cells

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Signals mediated by the chemokine CXCL12 and its receptor CXCR4 are involved in the progression of ovarian cancer through enhancement of tumor angiogenesis and immunosuppressive networks that regulate dissemination of peritoneal metastasis and development of cancer-initiating cells (CICs). In this study, we investigated the antitumor efficacy of a CXCR4 antagonist expressed by oncolytic vaccinia virus (OVV) against an invasive variant of the murine epithelial ovarian cancer cell line ID8-T. This variant harbors a high frequency of CICs that form multilayered spheroid cells and express the hyaluronan receptor CD44, as well as stem cell factor receptor CD117 (c-kit). Using an orthotopic ID8-T tumor model, we observed that i.p. delivery of a CXCR4 antagonist–expressing OVV led to reduced metastatic spread of tumors and improved overall survival compared with oncolysis alone. Inhibition of tumor growth with the armed virus was associated with efficient killing of CICs, reduced expression of ascitic CXCL12 and vascular endothelial growth factor, and decreases in i.p. numbers of endothelial and myeloid cells, as well as plasmacytoid dendritic cells. These changes, together with reduced recruitment of T regulatory cells, were associated with higher ratios of IFN-γ/IL-10 tumor-infiltrating T lymphocytes, as well as induction of spontaneous humoral and cellular antitumor responses. Similarly, the CXCR4 antagonist released from virally infected human CAOV2 ovarian carcinoma cells inhibited peritoneal dissemination of tumors in SCID mice, leading to improved tumor-free survival in a xenograft model. Our findings demonstrate that OVV armed with a CXCR4 antagonist represents a potent therapy for ovarian CICs with a broad antitumor repertoire. The Journal of Immunology, 2014; 193: 5327–5337.

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancies (1). Peritoneal dissemination is a common route of disease progression of ovarian cancer, which occurs by implantation of tumor cells onto the mesothelial lining in the peritoneal cavity (2, 3). Despite modest improvements in progression-free and median survival using adjuvant platinum and paclitaxel chemotherapy following cytoreductive surgery, overall survival rates for patients with advanced EOC remain disappointingly low (4). Preclinical and clinical studies suggest that tumor initiation and maintenance are attributed to a unique population of sphere-forming cells enriched in cancer-initiating cells (CICs) that critically contribute to ovarian cancer tumorigenesis, metastasis, and chemotherapy resistance (5, 6). The presence of CICs in ovarian tissue samples and cell lines was demonstrated in multiple studies (7–9), and several markers have been used for their identification, including CD117, CD44, CD133, aldehyde dehydrogenase isoform 1 (ALDH1), and, in some cases, CD24 (9–11). These CICs were shown to survive in some cases, CD24 (9–11). These CICs were shown to survive in some cases.
growth (20). CXCL12 produced by tumor tissue and surrounding stroma stimulates vascular endothelial growth factor (VEGF)-mediated angiogenesis (21) and the recruitment of endothelial progenitor cells from the bone marrow (BM) (22, 23). CXCL12 also was shown to recruit suppressive CD11b+Gr1+ myeloid cells and plasmacytoid dendritic cells (pDCs) at tumor sites (24–26) and induce intratumoral Treg localization (26, 27), which impede immune mechanisms of tumor destruction. Therefore, modulation of the CXCL12/CXCR4 axis in ovarian cancer could impact multiple aspects of tumor pathogenesis, including immune dysregulation. Several CXCR4 antagonists demonstrated antitumor efficacy in preclinical models and have been evaluated in early clinical trials (28–31). However, given the abundant expression of CXCR4 by many cell types, including those of the central nervous, gastrointestinal, and immune systems (32), the side effects of these antagonists need to be taken into consideration. Furthermore, the impact of soluble CXCR4 antagonists on the mobilization of CXCR4-expressing BM-derived stem and progenitor cells represents an additional concern, particularly when combined with chemotherapeutic agents, because of the potential for adverse effects on hematopoiesis (33, 34).

To overcome some of these concerns related to the systemic delivery of soluble CXCR4 antagonists, we designed a tumor cell–targeted therapy that delivered a CXCR4 antagonist expressed in the context of the murine Fc fragment of IgG2a via an oncolytic vaccinia virus (OVV–CXCR4–A–Fc) (35). To that end, the antagonist was cloned into the genome of the oncolytic vaccinia virus (OVV), where selective replication in cancer cells is associated with cellular EGFR/Ras signaling, thymidine kinase elevation, and type-I IFN resistance (36). We chose OVV as a delivery vector because the virus has evolved mechanisms for rapid cell-to-cell spread to distant tissues, strong lytic activity, and large transgene-encoding capacity and has proven safety in humans as a vaccine (36–38). The destructive nature of a poxvirus infection results in the release of several cellular and viral danger signals, leading to generation of inflammatory responses that ultimately overcome tumor-mediated immune suppression to clear the virus (35, 39, 40), while also mediating tumor destruction.

Previously, we demonstrated that OVV–CXCR4–A–Fc delivered i.v. to mice with orthotropic breast tumors results in higher intra-tumor concentration of the inhibitor than its soluble counterpart, and OVV–CXCR4–A–Fc exhibits increased efficacy over oncology alone (35). These results, together with those of the previous studies showing targeting of lung and colon CICs (37) and breast cancer stem-like cells (41) with OVVs, prompted us to investigate whether OVV–CXCR4–A–Fc could effectively eliminate metastatic ovarian cancer dissemination and enhance the pool of tumor-associated Ags for immunization. Using a highly tumorigenic variant of the murine epithelial ovarian cancer cell line ID8-T that harbors CD44+ and CD117+ CICs, we demonstrated that antitumor efficacy of i.p.-delivered OVV–CXCR4–A–Fc was multifaceted, resulting in a direct oncolysis of CICs, decreases in recruitment of suppressive elements promoting tumor vascularization, and stimulation of antitumor immunity monitored by the presence of humoral and cellular immune responses to Wilms’ tumor Ag 1 (WT1) expressed by ID8-T cells.

Materials and Methods

Animals and cell lines

Female C57BL/6 and C.B-17 IcrTac-Prkdc SCID mice, 6–8 wk of age, were obtained from Taconic Farms (Hudson, NY) and the Laboratory of Animal Resources at Roswell Park Cancer Institute, respectively. Experimental procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Roswell Park Cancer Institute. ID8 mouse ovarian epithelial cells were derived from spontaneous malignant transformation of C57BL/6 MOSE cells (42), whereas human CaOV2 cells were derived from an ascitic tumor obtained from a patient with primary stage III serous ovarian carcinoma (43). Human HuTK+ 143 fibroblasts, human cervical carcinoma HeLa cells (44), and the African green monkey cell line CV-1 (45) were obtained from the American Type Culture Collection (Manassas, VA).

Viruses

The pFU–Luc2–Tomato lentiviral vector encoding firefly luciferase (Luc) fused to the red fluorescent protein td-Tomato (L2T) was kindly provided by Dr. M. Clarke (Stanford University, Stanford, CA). All vaccinia viruses used in this study are of the Western Reserve strain with disrupted TK and VGF genes for enhanced cancer cell specificity. The generation and characterization of vaccinia virus expressing EGFP (OVV-EGFP) and OVV–CXCR4–A–Fc were described previously (35). For the generation of OVV expressing the Fc portion of murine IgG2a (OVV-Fc), the Fc fragment was obtained from the pFUSE–mlG2a–Fc2 vector (InvivoGen, San Diego, CA) and cloned into the SaIl and XbaI restriction enzyme sites of the pCB023 plasmid under control of the vaccinia synthetic early/late promoter PseI (46). The inserted fragment was flanked by portions of the TK gene that allowed for the homologous recombination of mFcIgG2a into the TK locus of parental VSC20 vaccinia virus. For these experiments, confluent wells of CV-1 cells were infected for 2 h at 37°C with 1.4 × 10^6 PFU VSC20 in 1.0 ml MEM-2.5% FCS. Supernatants were removed, and a liposomal transfection (Invitrogen, Carlsbad, CA) of pCB023-mFcIgG2a plasmid was performed according to the manufacturer’s protocol. Multiple plaques of the recombinant viruses were isolated on a monolayer of HuTK+ 143 fibroblasts by BrdU selection. The OVV–CXCR4–A–Fc, OVV-EGFP, and OVV-Fc viruses were amplified on HeLa cells, purified over a sucrose gradient, titrated, and used for in vitro and in vivo studies.

Self-renewal sphere-formation assay and viral infection

The sphere assay was performed as described (12). Briefly, tumor cells were plated in ultralow attachment plates (Corning, Corning, NY) in serum-free DMEM/F12 supplemented with 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 0.4% BSA (Sigma-Aldrich), 10 ng/ml basic fibroblast growth factor (In Vitrogen), and 20 ng/ml recombinant epidermal growth factor (Invitrogen) at a density of 1,000–10,000 viable cells/well. Sphere formation was assessed 12–14 d after seeding. For the viral infection, spheres were collected by gentle centrifugation, dissociated in trypsin-EpDNA solution, and incubated with OVV-EGFP at a multiplicity of infection (MOI) of 1 for 2 h in 5% CO2. Cells were washed, seeded in cultures and evaluated for EGFP expression by fluorescence microscopy after 12 h.

In vivo studies

Tumorigenicity of the bulk ID8 and ID8-T cell lines was assessed by measuring tumor formation after injection of different numbers of cells (2 × 10^3–5 × 10^5) s.c. into dorsal tissues or i.p. into C57BL/6 or SCID mice (n = 3 or 4/group). In some experiments, ID8-T tumor cells were sorted based on CD44 and/or CD117 expression prior to s.c. inoculation of CD44+CD117+, CD44+CD117−, CD44−CD117+, or CD44−CD117− cell populations (5 × 10^2–2 × 10^3 cells/injection in 50 μl PBS) into C57BL/6 mice. Engrafted mice were inspected biweekly for tumor appearance by palpation and subjected to bioluminescence imaging to quantify tumor burden using the Xenogen IVIS Imaging System (PerkinElmer, Waltham, MA) after injection of 200 μl Luciferin-D (150 mg/kg body weight; Biosynth International, Itasca, IL). For oncolytic virotherapy studies, C57BL/6 mice (n = 8–10/group) were injected i.p. with 5 × 10^7 ID8-T cells, whereas SCID mice (n = 8/group) were injected i.p. with 5 × 10^6 CaOV2 cells. Oncolytic virotherapy with OVV–CXCR4–A–Fc, OVV-Fc, or OVV-EGFP (10^7 PFU) was injected 7 d after seeding. For the viral infection, spheres were collected by gentle centrifugation, dissociated in trypsin-EpDNA solution, and incubated with OVV-EGFP at a multiplicity of infection (MOI) of 1 for 2 h in 5% CO2. Cells were washed, seeded in cultures and evaluated for EGFP expression by fluorescence microscopy after 12 h.

Flow cytometry

ID8 and ID8-T tumor cells were analyzed by staining of single-cell suspensions with rat mAbs against mouse CD117-allophycocyanin, CD44–
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Intracellular expression of WT1 was evaluated with anti-WT1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), followed by goat anti-mouse IgG-FITC (BD Pharmin- 
gen). All evaluations were performed on a FACSciCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). After gating on forward and side scatter parameters, 10,000 gated events were routinely acquired and analyzed using CellQuest software (Becton Dickinson Immunocytometry Systems). Sorting of CD117⁺CD44⁺, CD117⁻CD44⁻, CD117⁺CD44², and CD117⁻CD44² subsets of the ID8-T cell line was performed on a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA). ALDH1⁻ enzymatic activity was defined using the ALDEFLUOR kit (Stell Cell Technologies, Vancouver, BC, Canada), according to the manufacturer’s protocol.

The phenotypic analysis of tumor stromal cells and immune infiltrates (myeloid, endothelial cells, pDCs, Tregs, and lymphocytes) was performed on single-cell suspensions prepared from peritoneal fluids collected at the time the control mice developed abdominal swelling. The cells were stained with rat anti-mouse Abs: CD11b-allophycocyanin, Ly6G-PE, Ly6C-FITC, B220-allophycocyanin, CD11c-PE, CD45-allophycocyanin−Cy7, VEGFR-2-PerCP-Cy5.5, CD34-FITC, CD45-PE, CD117-PE, CD8-PECY5, IFN-γ-PE, IL-10-PE (all from BD Pharmin- 
gen), Fasp3-, Alexa Fluor 647 (eBioscience, San Diego, CA), and CD117-PE (Abcam, Cambridge, MA). The numbers of CD4⁺ and CD8⁺ T cells expressing IFN-γ or IL-10 and CD4⁺ T cells expressing Fasp3 were determined by intracellular staining using a BD Cytometric Cytperm kit (BD Pharmin- 
gen), according to the manufacturer’s protocol. For the analysis, immune cells were gated on CD45⁺ cells, and endothelial progenitor cells were gated on CD34⁺ cells. To determine the percentage of WT1⁺CD44⁺H-D⁺ tetramer–specific CD8⁺ T cells, lymphocytes obtained from axillary, brachial, and inguinal lymph nodes were incubated with LPS-matured WT1⁺CD44⁺H-D⁺ tetramer (RMPNPAPYL) peptide–coated dendritic cells (DCs) for 72 h in the presence of IL-2 (0.3 ng/ml), as described (48). The cells were washed and stained with rat anti-mouse CD8-PECY5 mAb and a PE-labeled WT1⁺CD44⁺H-D⁺ tetramer (MHC Tetramer Production Facility, Baylor College of Medicine, Houston, TX). Background staining was assessed using isotype control Abs (BD Pharmin- 
gen). Before specific Ab staining, cells were incubated with Fc blocker (anti-CD16/CD32 mAb) for 10 min and analyzed on a FACSciCalibur flow cytometer.

ELISA

The expression of CXCL12 and VEGF proteins in cell-free peritoneal fluids was analyzed by a CXCL12/SDF-1a ELISA Quantikine Kit (R&D Sys- tems) and a mouse VEGF-α ELISA kit (Antigenix America, Huntington Station, NY), respectively, according to the manufacturers’ instructions. To measure the levels of WT1-specific Abs after oncolytic virotherapy treatment, blood samples were collected by retro-orbital bleeding, and sera (1:100 dilution) were analyzed by ELISA with wells coated with 3 µg/ml WT1 peptide (AAPP Tec, Louisville, KY).

CTL assay

Splenocytes were cultured with WT1⁺CD44⁺H-D⁺ tetramer–coated DCs at a 20:1 ratio for 24 h, after which cells were split and cultured in medium sup- plemented with murine rIL-2 (0.3 ng/ml; BD Biosciences). The cytolytic activity of CTLs against ID8-T tumor cells was analyzed 5 d later by a 51Cr-release assay. The percentage of specific lysis was calculated as follows: (cpm experimental release – cpm spontaneous release)/cpm maximum release – cpm spontaneous release) × 100. Maximum cpm release was determined from the supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was de- termined from target cells incubated with medium only.

Statistical analyses

The statistical significance of the difference between groups was performed using the two-tailed Student t test assuming equal variance. Mixed-model ANOVA was used to compare differences in sphere formation, suscepti- bility of tumor cells to viral infection, metastatic dissemination, and immuno-suppressive networks of the tumor microenvironment between groups. The p values < 0.05 were considered statistically significant. Kaplan–Meier survival plots were prepared and median survival times were determined for tumor-challenged groups of mice. Statistical differences in the survival across groups were assessed using the log-rank Mantel– 
Cox method. Data are presented as arithmetic mean ± SD and were analyzed using JMP software (SAS Institute, Cary, NC) on a Windows-based platform.

Results

Characterization of invasive ID8-T ovarian tumor cells

The invasive ID8-T tumor-forming variant was isolated from ascites of ID8 tumor-bearing mice based on expression of CIC- 
associated markers and was subsequently transduced with L2T lentiviral vector encoding Luc fused to the red fluorescence protein td-Tomato. Flow cytometry analysis revealed that >80% of ID8-T cells expressed the hyaluronan receptor CD44 in contrast to ~30% of CD44⁺ cells in the parental ID8 culture (Fig. 1A). The expression levels of the stem cell factor receptor CD117 (c-kit) and CD24 Ag also were higher on ID8-T cells, whereas the numbers of cells positive for CD33 Ag or exhibiting ALDH1 activity remained relatively low in both cultures (Fig. 1A). Cells positive for both CD44 and CD117 Ags made up ~40% of ID8-T tumors; in contrast, <2% of these cells were found in ID8 culture, which contained mostly CD44⁺CD117⁻ cells (Fig. 1B). Consistent with a previous report of CD44 and CD117 expression on sphere-forming ovarian CICs (11), the double-positive ID8-T cells or those expressing CD117 Ag were more efficient at sphere formation than were their CD117⁻ counterparts (Fig. 1C). The bulk culture of ID8-T cells and its CD44⁺CD117⁻ subset had reduced numbers and sizes of spheres, whereas the double- negative cells formed spheres only sparsely, suggesting that decreased numbers of CD44⁺CD117⁻ cells may be responsible for the reduced sphere formation in the parental ID8 cell line (data not shown).

The tumorigenicity of both bulk cultures, as well as CD44⁺ and/or CD117⁺ subsets of ID8-T cells, was examined by inculat- ing exponentially smaller numbers of cells s.c. or into the perito- neal cavities of C57BL/6 syngeneic and SCID mice. As shown in Table I, a minimum of 5 × 10⁵ ID8 cells was required to initiate s.c. tumor growth in all inoculated mice within a 7-wk period, whereas injection of 10% of that number formed ID8-T tumors within a much shorter period of time. ID8-T cells were also more efficient than their parental counterpart in causing bloody ascites in C57BL/6 and SCID mice, with metastatic dissemination detected in the omentum, diaphragm, mesentery, and peritoneal wall, indicating that these cells recapitulate the progression of ovarian cancer seen in patients. The rates of s.c. tumor formation with CD44⁺CD117⁻ and CD44⁺CD117⁺ tumor cells were consis- tently higher compared with the CD117⁻ subset or the bulk culture (Table I). With injections of only 10,000 CD117⁺ cells, palpable tumors were detected in all challenged mice within a 3-wk period. At this number and within the same period of time, the double-negative cells did not form tumor, whereas the CD44⁺CD117⁻ subset displayed an intermediate phenotype with lower tumorigenicity (Table I). Altogether, these results indicate that the ID8-T cell line is enriched for CD44⁺CD117⁺ and CD117⁺ cells with CIC-like attributes compared with the parental ID8 culture.

Susceptibility of ID8-T tumor cells to vaccinia virus infection

We next investigated the susceptibility of both ID8 and ID8-T bulk cultures to OVV-EGFP infection (MOI = 1) by analyzing expression of EGFP in infected cells 12 h later. As shown in Fig. 2A, flow cytometry analysis revealed that the numbers of EGFP⁺ cells in ID8-T culture were almost 2-fold higher than those in the parental ID8 counterpart. More than 80% of infected cells were CD44⁺ in both cultures, but they differed with regard to CD117 expression. The latter Ag was present on~40% of vaccinia virus- infected ID8-T cells but was reduced nearly to background level in the ID8-infected culture (Fig. 2A). The results of this study sug- gest that the highly tumorigenic ID8-T cells are preferentially targeted by the virus. This observation was further supported by

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comparing OVV-EGFP infection in ID8-T adherent and spheroid populations. Analyses of both cultures under the fluorescence microscope revealed that, although approximately half of the adherent ID8-T tumor cells were infected with the virus, the majority of spheroid cells were highly positive for EGFP expression (Fig. 2B).

To evaluate the ability of vaccinia virus to target ovarian CICs in vivo and any improvement in cancer cell killing gained through expression of the CXCR4 antagonist by the virus, ID8-T cells (5 × 10^5) were injected i.p. into syngeneic C57BL/6 mice and treated 7 d later with OVV–CXCR4-A–Fc or OVV-Fc at 10^8 PFU/injection. OVV-Fc, rather than OVV-EGFP, was used as a control vector for the in vivo studies to account for any possible effects that could be attributed to the Fc portion of the CXCR4-A–Fc fusion protein. The viruses were delivered i.p., and progression of tumor growth was quantified by bioluminescence imaging (radiance) 8 d after the initiation of treatment, which roughly corresponds to the termination of viral replication.

### Table I. In vivo tumorigenicity of ID8 and ID8-T bulk cultures, as well as CD44+ and/or CD117+ and double-negative subsets of ID8-T cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Phenotype</th>
<th>Injection Route</th>
<th>Cell Dose (x10^3)</th>
<th>Tumor Formation</th>
<th>Latency Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID8</td>
<td>Bulk culture</td>
<td>s.c.</td>
<td>5</td>
<td>3/3</td>
<td>40/49/52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>5 × 10^3</td>
<td>1/3</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>5 × 10^6</td>
<td>4/4</td>
<td>61/65/67/70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>5 × 10^6</td>
<td>3/3</td>
<td>63/68/72</td>
</tr>
<tr>
<td>ID8-T</td>
<td>Bulk culture</td>
<td>s.c.</td>
<td>5 × 10^3</td>
<td>3/3</td>
<td>24/29/32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>2 × 10^4</td>
<td>2/3</td>
<td>37/39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>5 × 10^6</td>
<td>3/3</td>
<td>24/28/35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>5 × 10^6</td>
<td>3/3</td>
<td>44/47/49</td>
</tr>
<tr>
<td>ID8-T</td>
<td>CD44+CD117+</td>
<td>s.c.</td>
<td>1 × 10^4</td>
<td>3/3</td>
<td>18/20/22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>5 × 10^3</td>
<td>1/3</td>
<td>27</td>
</tr>
<tr>
<td>ID8-T</td>
<td>CD44-CD117+</td>
<td>s.c.</td>
<td>1 × 10^4</td>
<td>3/3</td>
<td>17/18/23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>5 × 10^3</td>
<td>2/3</td>
<td>26/29</td>
</tr>
<tr>
<td>ID8-T</td>
<td>CD44+CD117-</td>
<td>s.c.</td>
<td>1 × 10^4</td>
<td>2/3</td>
<td>27/50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>5 × 10^3</td>
<td>3/3</td>
<td>NA</td>
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<tr>
<td>ID8-T</td>
<td>CD44-CD117-</td>
<td>s.c.</td>
<td>1 × 10^4</td>
<td>3/3</td>
<td>44/48/54</td>
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<td>s.c.</td>
<td>4 × 10^3</td>
<td>1/3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>1 × 10^4</td>
<td>0/3</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a Bulk cultures or ID8-T tumor cells sorted based on expression of CD44 and CD117 Ags were injected s.c. or i.p. at different numbers into syngeneic C57BL/6 or SCID mice and monitored for tumor growth.

*b No. of cells/injection.

*c No. of tumors/no. of injections.

*d Time from injection to the first appearance of a palpable tumor or ascites.

*e Experiments were performed in SCID mice.

NA, not applicable.
in vivo (data not shown). As shown in Fig. 2C and 2D, the tumor burden after OVV–CXCR4-A–Fc treatment was significantly reduced compared with control and OVV-Fc–treated mice (Fig. 2D; \( p = 0.007 \) and \( p = 0.015 \), respectively), which also was reflected in lower numbers of tumor cells recovered from the peritoneal cavities of the treated mice (data not shown). There also were phenotypic differences among tumor cells in control and virally treated mice. As shown in Fig. 2E, percentages of CD44+CD117+ and CD44−CD117+ ID8-T cells were reduced to almost background levels after OVV–CXCR4-A–Fc treatment and were significantly lower compared with those in OVV-Fc–treated mice (\( p = 0.003 \) and \( p = 0.01 \)) and control animals (\( p < 0.001 \) and \( p = 0.005 \)). OVV-Fc treatment also reduced the numbers of CD44+CD117+ and CD44−CD117+ cells compared with the control tumor (Fig. 2E; \( p < 0.05 \)), whereas both viruses were less effective in eliminating CD44−CD117− and double-negative tumor cells. Because CXCR4 was expressed on the surface of CD117+ sphere-forming cells (Supplemental Fig. 1), the greater in vivo decrease in CD117+ CICs by OVV-CXCR4 compared with that in OVV-Fc-treated tumors might be attributed to interference of the CXCR4-A–Fc antagonist, released from virally infected cells, with the CXCL12/CXCR4-signaling axis on these cells and/or Ab-dependent cell–mediated cytotoxicity– and complement-dependent cytotoxicity-mediated killing (35). These findings, together with the increased percentage of double-negative tumor cells in the virally

FIGURE 2. Susceptibility of ID8-T tumor cells to vaccinia virus infection. (A) ID8 and ID8-T cells were infected with OVV-EGFP (MOI = 1) and analyzed by flow cytometry 12 h later to determine expression of EGFP in CD44+ and CD117+ tumor cells (right panels). (B) ID8-T cells were cultured as a monolayer or in serum-free medium for 12 d before infection with OVV-EGFP (MOI = 1). The expression of EGFP in infected cells was examined under an immunofluorescence microscope 12 h later. Scale bars, 25 μm. (C) ID8-T cells (5 × 10⁶ cells) were injected i.p. into syngeneic C57BL/6 mice and treated with OVV-Fc or OVV–CXCR4-A–Fc (10⁸ PFU) after 7 d. The viruses were delivered i.p., and the effect of the treatments on tumor growth was determined by bioluminescence imaging 8 d later. (D) Quantification of the bioluminescent signals from the tumor regions. Error bars represent the SD of the mean of three experiments with three or four mice/group. (E) ID8-T tumor cells were isolated from peritoneal cavities 8 d after the treatment and analyzed by flow cytometry for expression of CD117 and CD44 Abs. Percentages of CD117+CD44+, CD117+CD44−, CD117−CD44+, and CD117−CD44− are presented as mean ± SD. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
treated mice compared with controls \( (p < 0.01) \), suggest selective targeting of CICs by OVV–CXCR4-A–Fc in vivo.

OVV–CXCR4-A–Fc inhibits i.p. dissemination of ID8-T tumor and improves overall survival

Although the single oncolytic virotherapy treatment led to a significant reduction in tumor growth, the presence of residual tumors prompted us to examine whether additional injections of the virus, repeated twice at a 1-wk interval (Fig. 3A), would lead to improved overall survival. Fig. 3B shows that OVV–CXCR4-A–Fc treatment resulted in an extended survival compared with control treatment \( (p < 0.001) \) or treatment with OVV-Fc \( (p = 0.002) \). At the time that the control mice were sacrificed because of extensive tumor burden associated with the development of bloody ascites (Fig. 3C, 3D), the tumor load was significantly reduced in OVV–CXCR4-A–Fc–treated mice \( (p = 0.005) \), and they had no evidence of ascites. These mice also had very small omental tumors and significantly reduced numbers of metastatic nodules (>5 mm) in the peritoneal cavities compared with their control and OVV-Fc–treated counterparts (Fig. 3E; \( p = 0.005 \) and \( p = 0.024 \), respectively). In control mice, the metastatic nodules were present on the omentum, mesentery, diaphragm, and peritoneal wall. At the time of the analysis, tumor growth in OVV–CXCR4-A–Fc–treated mice was located primarily in the omentum, with sporadic metastatic lesions on diaphragm and peritoneal wall (Fig. 3F). The metastatic dissemination was more prominent after treatment with OVV-Fc, although it was still lower than in control mice \( (p = 0.049) \). Similar results were obtained using a xenograft model of human CAOV2 ovarian carcinoma in SCID mice. Intraperitoneal injection of \( 2.5 \times 10^7 \) PFU of OVV–CXCR4-A–Fc into CAOV2-bearing mice contributed to inhibition of tumor growth and metastatic dissemination, leading to tumor-free survival in ~20% of CAOV2-bearing mice (Supplemental Fig. 2).

OVV–CXCR4-A–Fc decreases levels of ascitic CXCL12 and VEGF, as well as recruitment of endothelial progenitor cells, neutrophils/granulocytic-myeloid–derived suppressor cells, and pDCs

Because the CXCR4 receptor for CXCL12 chemokine is one of the key stimuli involved in signaling between tumor cells and their
microenvironment, we next investigated whether inhibition of peritoneal dissemination of ID8-T tumor after targeting the CXCL12/CXCR4-signaling axis through an oncolytic virus also would result in changes within the tumor microenvironment. ELISA analyses of CXCL12 protein levels in peritoneal fluids harvested from tumor-bearing mice at the time when the control mice developed abdominal swelling consistent with ascites production, which roughly corresponded to 40 d after tumor challenge, revealed ~4-fold higher levels of the chemokine in control and OVV-Fc–treated tumors compared with their OVV–CXCR4-A–Fc–treated counterparts (Fig. 4A; $p < 0.001$ and $p = 0.004$, respectively). Changes in CXCL12 expression paralleled those of VEGF in ascitic fluids, where the levels of VEGF were significantly reduced after treatment with the armed virus compared with control and OVV–Fc–treated mice (Fig. 4A; $p < 0.001$). VEGF, whose expression is affected by CXCL12 (49), is pivotal in tumor angiogenesis (50, 51) and is associated with poor clinical outcome in patients with ovarian cancer (52). Thus, the higher CXCL12 and VEGF levels in peritoneal fluid together with intense neo-vascularization, manifested by an early sign of bloody ascites formation in OVV–Fc–treated tumors (Fig. 3C), suggest a potential angiogenic “rebound” in these mice compared with animals treated with OVV–CXCR4-A–Fc. This possibility was supported by other findings demonstrating that binding of CXCL12 to CXCR4 expressed on circulating endothelial progenitor cells (EPCs), neutrophils/granulocytic-myeloid–derived suppressor cells (G-MDSCs), and pDCs triggers migration of these cells to tumor sites (23–25, 53, 54).

Parallel analyses of the recruitment of EPCs to control and OVV-treated tumors examined by immunofluorescence staining of single-cell suspensions with mAbs specific for CD34, CD117, and VEGFR-2 revealed that their accumulation in the peritoneal cavity after treatment with the armed virus was significantly diminished compared with their control and OVV-Fc–treated counterparts (Fig. 4C; $p = 0.0009$ and $p = 0.013$, respectively). We also observed that the percentage of EPCs in control tumors was higher than that in OVV-Fc–treated mice ($p = 0.005$), despite comparable levels of CXCL12 and VEGF in both groups. Although the reason for this discrepancy is unknown, it is possible that other types of cells with proangiogenic activities, including neutrophils/G-MDSCs and pDCs, could be recruited to the tumor after infection with the unarmed virus and promote angiogenesis by producing VEGF and angiogenic cytokines, respectively (55, 56).

Because neutrophils/G-MDSCs are one of the first cell types recruited to sites of infection (57), single-cell suspensions prepared from the control and virally treated animals were analyzed for the expression of CD11b, Ly6G, and Ly6C markers by flow cytometry. We focused on cells with high expression of CD11b and Ly6G Ags and low Ly6C levels, because this phenotype represents a population of granulocytes, including neutrophils and G-MDSCs (58). Consistent with the notion that changes mediated by oncolytic virotherapy within tumors may attract activated neutrophils (59), the percentages of CD11b$^+$Ly6ChighLy6G$^+$ cells in OVV–Fc–treated tumors were comparable with those in controls (Fig. 4E), despite lower tumor burden (Fig. 3C). In contrast, the accumulation of neutrophils/G-MDSCs in OVV–CXCR4-A–Fc–treated tumors was significantly reduced compared with their control and OVV-Fc–treated counterparts (Fig. 4D; $p = 0.04$ and $p = 0.022$, respectively). A similar profile of responses was observed in the recruitment of CXCR4$^+$ pDCs (B220$^{high}$Ly6ChighCD11c$^{low}$), which are known to enhance tumor angiogenesis through production of IL-8 and TNF-α (56) and contribute to the tumor immunosuppressive network through induction of IL-10–expressing CD8$^+$ T cells (60). Because migration of pDCs to the tumor sites is mediated by CXCL12 (25) and their expansion occurs during in vivo infection with vaccinia virus (61), the recruitment of pDCs in control and OVV-Fc–treated tumors were comparable and >3-fold higher than those in their OVV–CXCR4-A–Fc–treated counterparts (Fig. 4E; $p = 0.004$ and $p = 0.01$, respectively). Altogether, these results suggest that the effect of virally delivered CXCR4 antagonist on the CXCL12/CXCR4-signaling axis prevails over the inflammatory capacity of the oncolytic virus to recruit neutrophils/G-MDSCs and pDCs to the tumor microenvironment.

Inhibition of tumor-immunosuppressive networks by OVV–CXCR4-A–Fc is accompanied by the induction of antitumor immune responses

The inhibition of peritoneal dissemination of ID8-T tumor after OVV-CXCR4 treatment could be accounted for by both a direct cytotoxic effect of the virus, as well as induction of antitumor immunity because of the ability of vaccinia virus to break Treg-
mediated tolerance through TLR-dependent and -independent pathways (39, 40). This effect could be augmented by release of the CXCR4-A–Fc fusion protein from OVV–CXCR4-A–Fc–infected tumor cells because CXCL12 induces intratumoral localization of CD4+CD25+Foxp3+ Tregs in ovarian carcinoma (26, 27). In concordance with these findings, flow cytometry analysis revealed that treatment with OVV–CXCR4-A–Fc resulted in significantly lower percentages of tumor-infiltrating Tregs compared with control or OVV-Fc treatment (Fig. 5A, \( p = 0.001 \) and \( p = 0.03 \), respectively). This also could contribute to significantly higher ratios of IFN-\( \gamma \)- and IL-10–producing CD8+ TILs in OVV–CXCR4-A–Fc–treated tumors compared with those in their control (\( p = 0.007 \)) and OVV-Fc–treated counterparts (Fig. 5B; \( p = 0.048 \)). The changes were even more prominent with regard to the accumulation of IFN-\( \gamma \)- and IL-10–producing CD8+ TILs, as reflected by 2-fold increases in these ratios after OVV–CXCR4-A–Fc treatment compared with control or OVV-Fc treatment (Fig. 5B; \( p = 0.002 \) and \( p = 0.011 \), respectively), suggesting that delivery of the armed virus is able to alter the inflammatory status of the tumor microenvironment in favor of immune activity over immune suppression.

To investigate whether the changes in the virally treated tumor microenvironment were associated with the generation of spontaneous antitumor immunity, sera were collected from tumor-bearing mice before viral challenge and at the time of ascites development in control mice. The sera specimens (dilution of 1:100) were analyzed by ELISA for the presence of Abs to WT1 Ag as a surrogate marker of the treatment-induced antitumor immune responses. As shown in Fig. 5C, tumor-bearing control mice were unable to mount antitumor humoral responses. In contrast, WT1-specific serum Abs were present in OVV–CXCR4-A–Fc–treated mice, with 3-fold increases compared with those in their OVV-Fc–treated counterparts (Fig. 5C; \( p = 0.014 \)). The Ab against WT1 was predominantly IgM, with IgG detected in 25% of OVV–CXCR4-A–Fc–treated mice with small tumor burden (data not shown).

Because new approaches for increasing the size and breadth of tumor-specific effector and memory pools of T cells are needed to enhance the efficacy of oncolytic virotherapy, we investigated the presence of oncolytic virotherapy–induced CD8+ T cells to the WT1126–134 epitope (RMFPNAPYL) with H2-D\( b \)-binding motif in the same group of mice that were analyzed for humoral responses.

**FIGURE 5.** OVV–CXCR4-A–Fc inhibits tumor immunosuppressive networks and promotes induction of antitumor immune responses. (A) The numbers of Tregs in ascites-derived tumors of control and virally treated mice were analyzed by flow cytometry after staining of single-cell suspensions with anti-CD45–allophycocyanin, anti-CD4–PE, anti-CD25–FITC, and anti-Foxp3–Alexa Fluor 647. (B) The ratios of IFN-\( \gamma \)- and IL-10–producing CD8+ TILs were determined by flow cytometry after intracellular staining with rat mAbs against mouse IFN-\( \gamma \)-PE or IL-10–PE, followed by CD4-PECy5 or CD8-PECy5. Background staining was assessed using isotype-control Abs. Results are mean ± SD of three or four independent experiments. (C) Sera were collected from tumor-bearing mice, before viral challenge and at the time that the control mice developed ascites, and analyzed by ELISA for the presence of Abs to WT1 Ag. All samples were analyzed in triplicates with serum dilution of 1:100. (D) The percentage of WT1126–134 tetramer–specific CD8+ T cells was determined in lymphocytes obtained from axillary, brachial, and inguinal lymph nodes isolated from the same group of animals that were analyzed for humoral responses. The isolated cells were incubated with LPS-matured WT1126–134 peptide–coated DCs for 72 h in the presence of IL-2, washed, and stained with anti-CD8-PE–Cy5 or PE–labeled H-2D\( b \)-restricted WT1126–134 tetramer. All evaluations were performed on a FACSCalibur flow cytometer. After gating on forward and side scatter parameters, 10,000 gated events were routinely acquired and analyzed using CellQuest software. (E) ID8-T–specific CTL responses. CD8+ splenocytes from untreated, OVV-Fc–treated, or OVV–CXCR4-A–Fc–treated tumor-bearing mice were cultured with WT1126–134 peptide–coated DCs at a 20:1 ratio, as described in Materials and Methods. The CTL activities against ID8-T cells were analyzed in a standard \([^{51}Cr]\)-release assay using the indicated E:T ratios. All determinations were made in triplicate samples, and SD was <10%. Data are mean ± SD of two independent experiments. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
To this end, cells isolated from the lymph nodes of control and virally treated mice were assessed, by flow cytometry, for the presence of CD8+ T cells able to bind WT1\textsubscript{126-134}/H2-D\textsuperscript{b} tetramers after 72 h of incubation with WT1\textsubscript{126-134} peptide–coated DCs. Fig. 5D shows that the percentage of CD8+ T cells specific for WT1\textsubscript{126-134}/H2-D\textsuperscript{b} epitope was nearly 2-fold greater after OVV–CXCR4-A–Fc therapy compared with OVV-Fc treatment (2.5 ± 0.5% versus 1.6 ± 0.3%; p = 0.04), whereas no WT1\textsubscript{126-134}/H2-D\textsuperscript{b} tetramer+ cells were detected in control mice. Additionally, the robust proliferation of splenocytes in response to stimulation with WT1\textsubscript{126-134} peptide–coated DCs was detected in cultures derived from tumor-bearing mice treated with OVV-Fc or OVV–CXCR4-A–Fc (data not shown). The proliferative responses were associated with the presence of antitumor CTL activities against ID8-T cells, as monitored 5 d later by a standard 4-h [\textsuperscript{51}Cr]-release assay (Fig. 5E).

**Discussion**

Ovarian CIC–mediated self-renewal, aggressive neovascularization, resistance to chemo- and radiotherapy, and marked local and systemic immunosuppression all contribute to current treatment inadequacy and tumor recurrence (9, 10). Thus, the curative potential of therapies against ovarian cancer hinges on eradicating CICs, in addition to countering the tumor immunosuppressive network (62). Cancer stem cell identification was largely based on primary cells, as well as early passage of cell lines in a mouse xenograft model (7, 11, 63). However, the majority of serum-cultured cell lines does not recapitulate the genotype and phenotype of ovarian cancer and, therefore, has limitations with regard to translating therapies to the clinic. The available immunocompetent EOC models to evaluate the multitude of therapies, especially those involving immunotherapy, rely primarily on ID8 cells (42), which are characterized by a slow growth rate and low numbers of CD117- and CD44-expressing cells. The highly invasive ID8-T preclinical model described in this article provides a means of investigating therapeutic impact on multiple aspects of ovarian cancer, including CICs, while also maintaining important pathophysiological characteristics of human ovarian tumors. Using this model, we demonstrated that OVV–CXCR4-A–Fc treatment of mice challenged with ID8-T tumor resulted in reductions in i.p. numbers of CD44\textsuperscript{+}CD117\textsuperscript{-} and CD44\textsuperscript{-}CD117\textsuperscript{+} CICs associated with increased survival. Specifically, we showed that the armed OVV was highly efficacious in treating ID8-T tumor, and its multifaceted activities were associated with enhanced infection and killing of CICs, a reduction in the tumor immunosuppressive network, and induction of antitumor humoral and cellular responses.

From a clinical perspective, our findings illustrating the contribution of OVV–CXCR4-A–Fc to targeting CICs, as well as their supportive microenvironment, may have significant therapeutic implications. In vitro analyses of tumor cells recovered from the peritoneal cavity after therapy with OVV–CXCR4-A–Fc revealed almost complete depletion of CD117\textsuperscript{+}CD44\textsuperscript{+} and CD117\textsuperscript{-}CD44\textsuperscript{-} cells with sphere-forming ability, although few of the remaining cells or CD44\textsuperscript{-}CD117\textsuperscript{-} and double-negative subsets could account for subsequent tumor regrowth. For example, the CD44\textsuperscript{-}CD117\textsuperscript{+} population with reduced viral clearance compared with CICs and “intermediate” ability to grow tumors at lower cell numbers could be responsible for the tumor recurrence. It is also possible that some ID8-T cells escaped the infection as a result of inadequate dissemination of the virus in the peritoneal cavity and/or the induction of antiviral immunity, particularly after the second viral injection, which could neutralize the additional treatments (64). Alternatively, some tumor cells could be refractive to viral infection. Several lines of evidence indicate that CICs encompass progenitor and aneuploid populations of tumor cells with microenvironmentally controlled switch between proliferation and quiescence (65–67). During the switch, quiescent CICs would undergo intermittent divisions, leading to self-renewal and generation of proliferating progenitors that give rise to the bulk tumors, as well as progenitor clones that constitute dormant subsets within tumor (65). Because cell cycle modifications are among the many mechanisms involved in controlling the dormant/CIC state of the tumor (66, 67), differences in cell cycle phases within euploid and aneuploid fractions may contribute to different susceptibility to vaccinia virus infection during treatment. Thus, it becomes increasingly important to exploit the effect of the virally delivered CXCR4 antagonist on the putative niche that nurtures CICs and their dormant progeny as a target for the oncolytic virotherapy.

Ovarian cancer is a heterogeneous disease with histologically defined subtypes, and reports showed that CICs are involved in drug resistance and cancer recurrence (10), indicating that advances in oncolytic virotherapy require effective direct lysis of CICs and manipulation of the tumor microenvironment that contributes significantly to tumorigenesis (68). Although oncolytic viruses have great potential for the treatment of tumors using direct cytotoxic and immune-stimulating mechanisms, and they have progressed to phase III clinical trials in patients (69), there is a limited understanding of the viral interaction with different subsets of tumor cells, as well as different elements of tumor stroma. In this study, we demonstrated that targeting the CXCL12/CXCR4 migratory axis with the virally expressed CXCR4 antagonist inhibited intratumoral accumulation of cancer-associated suppressive factors and cells, including EPCs and immunosuppressive MDSCs, pDCs, Tregs, and IL-10–producing CD4+ and CD8+ T cells, which had a significant therapeutic impact on metastatic dissemination of the tumor. Our findings also suggest that oncotherapy with the armed OVV may enhance the induction of anti-CIC immune responses by increasing the pool of tumor-associated Ags released from the virally infected tumors. In addition, because vaccinia virus–based vaccines were shown to elicit innate immunity through the TLR2/MyD88-dependent pathway and TLR-independent production of IFN-\(\beta\) (40), the ability of the virus to provide persistent TLR signals for immunotherapy in a setting of established tolerance, together with the CXCR4 antagonist–mediated reduction in intratumoral immunosuppressive elements, may produce a more permissive environment for anti-tumor immunity.

The induction of anti-WT1 Ab and CD8+ T cell responses during oncolytic virotherapy treatment has important implications for immunotherapy, because WT1 is one of the immunogenic tumor Ags expressed at high levels in a variety of human neoplasms, including EOC (69, 70). WT1 encodes a zinc-finger protein that plays a crucial role in the normal development of several organs (71), and it is essential for repression of the epithelial phenotype in epicardial cells and during embryonic stem cell differentiation through direct transcriptional regulation of genes encoding Snail and E-cadherin (72). Because both genes are mediators of epithelial–mesenchymal transition (72), which is a key developmental program that is often activated during cancer invasion and development of CICs (18, 73), expression of WT1 in spheres isolated from ID8-T cells (data not shown) suggests that it may serve as a target for T cell–mediated activity. Previous studies demonstrated that DCs pulsed with the lysates of CIC-enriched populations from histologically distinct murine tumors conferred antitumor immunity that was associated with the induction of humoral and cellular responses that directly targeted CICs via complement-dependent cytotoxicity and CTLs, respectively (74). In our studies, WT1-specific Abs could not have been directly involved in tumor cell lysis because WT1 is expressed intracel-
ularly. However, the Abs could form immune complexes with WT1 protein released from lysed tumor cells and facilitate enhanced Ag presentation to FcRs on DCs (75). In contrast, the lysis of tumor cells by CTLs may extend the remission period or even lead to tumor-free survival, particularly if some of these activities are directed against CICs. Consistent with this notion, ∼10% lysis at a 100:1 E:T ratio was detected in cultures of spheroid cells incubated overnight with IFN-γ for stimulation of MHC class I expression before the CTL assay (data not shown), stressing the need for additional studies to facilitate a more effective killing of these cells. Altogether, the presented mechanism of inhibition of pathways promoting tumor growth is likely applicable to different cancer types and potentially can unravel novel therapeutic avenues that efficiently target CICs to minimize the risk for tumor recurrence in cancer patients.

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References


