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14. ABSTRACT

The overall objective of this research project was to concomitantly reprogram the ovarian tumor microenvironment (TME) and tumor-specific T cell responses, while minimizing the potential for systemic toxicities. Because the CXCR4 receptor/CXCL12 chemokine signaling pathway triggers multiple adverse effects on ovarian cancer progression, we have developed a clinically translatable, oncolytic vaccinia virus expressing a CXCR4 antagonist (OVV-CXCR4-A) for reprograming the TME, and for sensitizing the tumor for treatment with dendritic cell (DC) vaccines and a novel adoptive T cell therapy. Using an orthotopic ID8-T ovarian tumor model in syngeneic mice, we demonstrated that intraperitoneal delivery of a CXCR4 antagonist-expressing virus was more efficacious against ID8-T tumor than a systemic injection of the armed vector or soluble antagonists. We also found that intratumoral delivery of the CXCR4-A-armed virus reduced tumor load and the immunosuppressive network in the TME, leading to increased efficacy of DC vaccines as well as infiltration of tumor antigen-specific $CD8^+$ T cells due to upregulated pathways of T cell trafficking.

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1. INTRODUCTION:

The overall goal of our research is to generate robust and long-lasting tumor-specific T cell responses for durable tumor regression in patients with chemotherapy-resistant ovarian cancer (OC). Although immunotherapy using immune checkpoint inhibitors (ICI), adoptive T cell transfer (ACT), or oncolytic viruses (OV) have generated remarkable results in several tumor types (e.g. melanoma, NSCLC), long-term tumor control has been infrequent in patients with OC (1-3). Previous studies have identified key stumbling blocks underpinning the limited anti-tumor efficacy of immunotherapy in OC (4-10), including: (i) insufficient expansion of tumor antigen-specific T cells, (ii) recruitment of CXCR4-expressing T regulatory cells (Tregs) and myeloid derived suppressor cells (MDSC) via tumor CXCL12 production, (iii) severe dysfunction of tumor infiltrating T lymphocytes (TILs) often by PD1 upregulation, (iv) low intrinsic tumor immunogenicity partially dependent on reduced tumor mutation burden and IFN-β production, (viii) insufficient recruitment of intratumoral dendritic cell (DC) populations capable of cross-presenting tumor antigens; and (ix) tumor "vascular checkpoint" characterized by disorganized and tortuous tumor vasculature lacking adequate flow dynamics to support trafficking of tumor-specific T cells. Because signals mediated by the chemokine CXCL12 and its receptor CXCR4 are involved in the progression of OC through enhancement of tumor angiogenesis and immunosuppressive networks that regulate dissemination of peritoneal metastasis, we investigated the antitumor efficacy of a CXCR4 antagonist against an invasive variant of the murine epithelial OC cell line ID8-T. The CXCR4 antagonist was delivered as a soluble protein (sCXCR4-A) or expressed by the oncolytic vaccinia virus (OVV-CXCR4-A) (11) to investigate whether targeting this axis in ID8-T tumor-bearing mice would inhibit tumor growth by the development of antitumor immunity though inhibition of suppressive elements in the tumor microenvironment (TME). Furthermore, since a delivery mode influences accumulation of the CXCR4-A antagonist in tumors and systemic tissues (12), we also analyzed the effect of intravenous (i.v.) and intraperitoneal (i.p.) injections of OVV-CXCR4-A on the induction of tumor-specific T cell responses, efficacy of dendritic cell (DC) vaccines, and adoptively transferred T cells. We **hypothesized** that the extent of tumor destruction by oncolytic virotherapy engineered to express the CXCR4-A antagonist and inhibition of the immunosuppressive network would affect the magnitude and duration of tumor-specific immune responses in the TME and control of tumor progression.

The following is a detailed account of our progress made for tasks outlined in the original SOW.

2. KEY WORDS:

CXCR4 antagonist, Oncolytic vaccinia virus, Ovarian tumor, Tumor microenvironment, Immunosuppression, Adoptive T cell transfer, Dendritic Cell Vaccine

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Aim 1. We compared inhibition of tumor growth and kinetics of recruitment of immunosuppressive cells to metastatic ovarian ID8-T tumors during treatments of tumor-bearing syngeneic mice with soluble and OVV-expressed CXCR4-A antagonists.

Aim 2. We analyzed the effect of soluble versus OVV-delivered CXCR4-A antagonist on the level and duration of Wilm'tumor antigen 1 (WT1)-DC vaccine-induced immune responses in tumor-bearing mice.

Aim 3. We examined the mechanisms of action associated with migration of tumor-specific T cells to tumor sites after ACT.

What was accomplished under these goals?

Body (from Original SOW): Specific Aim 1. Inhibition of tumor growth and recruitment of immunosuppressive cells to ID8-T tumors during treatment of tumor-bearing mice with soluble or tumor-targeted CXCR4-A antagonist.

- **Inhibition of ID8-T tumor progression after i.v. or i.p. injection of CXCR4-A delivered as a soluble antagonist or by oncolytic virotherapy.**

We first assessed the impact of i.v. or i.p delivery of soluble and virally-delivered CXCR4-A, expressed in-frame with the murine Fc fragment of IgG2a (OVV-CXCR4-A), in C57BL/6 mice challenged i.p. with a highly metastatic syngeneic ovarian cancer cell line (ID8-T). The treatment was initiated 10 days after tumor challenge and consisted of a single injection [10^8 plaque-forming units (PFU)/mouse] of OVV-CXCR4-A or control virus (OVV). To determine the contribution of the antagonist alone to controlling tumor growth, additional tumor-bearing mice were treated for 7 days with sCXCR4-A (10 μg/injection) delivered i.v. or i.p. or were injected with RPMI-1640 medium (control mice).

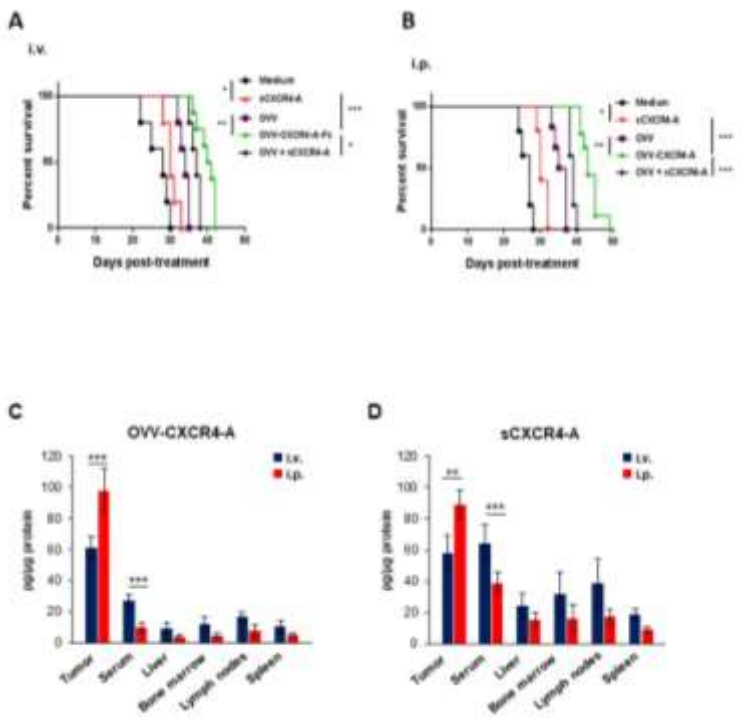

Figure 1 Inhibition of ID8-T tumor growth and accumulation of the CXCR4-A antagonist in peritoneal washes, sera and lymphoid organs after i.v. or i.p. delivery of OVV-CXCR4-A and sCXCR4-A. C57BL/6 female mice (n = 6-10 mice per group) were challenged i.p. with $3x10^5$ ID8-T tumor cells and treated with sCXCR4-A (10 μg/injection for 7 days), OVV or OVV-CXCR4-A (10^8 PFU), or OVV and sCXCR4 combination injected i.v. (**A**) or i.p. (**B**) 10 days after tumor challenge. Control mice were treated with medium. Tumor progression was monitored by bioluminescence. Kaplan-Meier survival plots were prepared and significance was determined using the log-rank method. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**C, D**) Accumulation of the sCXCR4-A antagonist in peritoneal washes, sera and lymphoid organs of tumor-bearing mice after i.v. or i.p. delivery of OVV-CXCR4-A or sCXCR4-A. Concentrations of CXCR4-A were determined on day 8 after treatments by ELISA after normalization to total protein content. Data are presented as the mean ± SD of five mice per group. **$P < 0.01$, ***$P < 0.001$.

Inhibition of tumor growth, quantified by bioluminescence imaging, revealed rapid tumor progression in untreated control mice (Fig. 1A,B), with animals reaching humane endpoint within 4 weeks of challenge. Systemic delivery of OVV-CXCR4-A reduced tumor growth and extended survival compared with untreated controls ($P < 0.001$) or animals treated with the unarmed virus ($P = 0.002$). On the other hand, systemic injection of sCXCR4-A demonstrated only modest effects in controlling tumor spread and extended survival by ~1 week compared to control, tumor-bearing mice. The antitumor effects of the virus or soluble antagonist were more pronounced after the i.p. treatment (Fig. 1B). Intraperitoneally-delivered OVV-CXCR4-A controlled tumor growth for 4-5 weeks, after which period tumor progressed, extending the survival by over 14 days compared to mice treated with sCXCR4-A ($P < 0.001$; Fig. 1B), or by ~10 days compared to the OVV-treated counterparts. A combination of the control virus and sCXCR4-A delivered either i.v. or i.p. was more efficacious in reducing tumor growth (Fig. 1A,B) and increased survival compared to each treatment alone ($P < 0.05$; Fig. 1A,B). The combination however did not achieve higher efficacy compared to that of a single treatment with OVV-CXCR4 (Figure 1A,B). This could be due to variations in distribution of sCXCR4-A in the TME after injections compared to close contact of the antagonist with tumor stroma and cancer cells after being released from OVV-CXCR4-A-infected cancer cells. Differences in the level and physical contact of sCXCR4-A with cancer cells could directly affect tumor growth through induction of apoptosis after binding to CXCR4-expressing ID8-T cells followed by phagocytosis of tumor cell debris by DCs, a process that is required for induction of antitumor immune responses (13). Thus, the more efficacious inhibition of ID8-T tumor growth by i.p. delivery of the antagonist either by the virus or in a soluble form could be associated with higher concentrations of sCXCR4-A in the tumor compared with systemic delivery as measured on day 8 after treatment ($P < 0.01$; Figure 1C,D). The i.p. treatment also resulted in

background levels of the antagonist in sera or other organs, which contrasted with ~2-fold higher levels of sCXCR4-A detected in sera and lymphoid organs of mice after systemic delivery.

- ***In vivo* pathogenicity of the CXCR4 antagonist determined by a complete blood count (CBC) and formalin fixed tissues**

We have analyzed changes in the CBC profile in tumor-bearing mice to evaluate the off-target effect of the oncolytic virotherapy delivered alone or in combination with the CXCR4 antagonists on the bone marrow (BM) pathology. The analysis was performed on heparinized peripheral blood samples collected from mice before (as a baseline level) and on days 8, 15, and 30 after each treatment. We conducted the study only after a systemic delivery of the virus and the CXCR4 antagonist because of higher concentrations of the CXCR4-A protein in sera compared to those measured after i.p. injection ($P = 0.015$) and accumulation of the antagonist in several organs, in contrast to only background levels measured after the i.p. treatment. The results of the CBC analyses depicted in Fig. 2A-C showed, except for small fluctuations, no changes in numbers of red blood cells and platelets during the 30-day post-treatment period. We detected increases in leukocyte counts on days 8 after each treatment, which persisted for approximately one week before returning to the baseline numbers. The soluble CXCR4-A fusion protein and the commercially available AMD3100 antagonist of the CXCR4 receptor increased numbers of leukocytes by ~10% on days 8 and 15. The changes in white blood cell (WBC) counts were more pronounced after virotherapy treatments and were on average 30% higher compared to the baseline, which is in line with the induction of inflammatory responses. Similarly, examination of formalin-fixed tissues of spleen, inguinal lymph node, BM, liver, kidney, and heart embedded in paraffin and stained with H&E performed 30 days post-treatment revealed no organ damage after systemic or i.p. deliveries of the virus alone or in combination with the soluble CXCR4 antagonists (data not shown), indicating no long-term pathogenic effect.

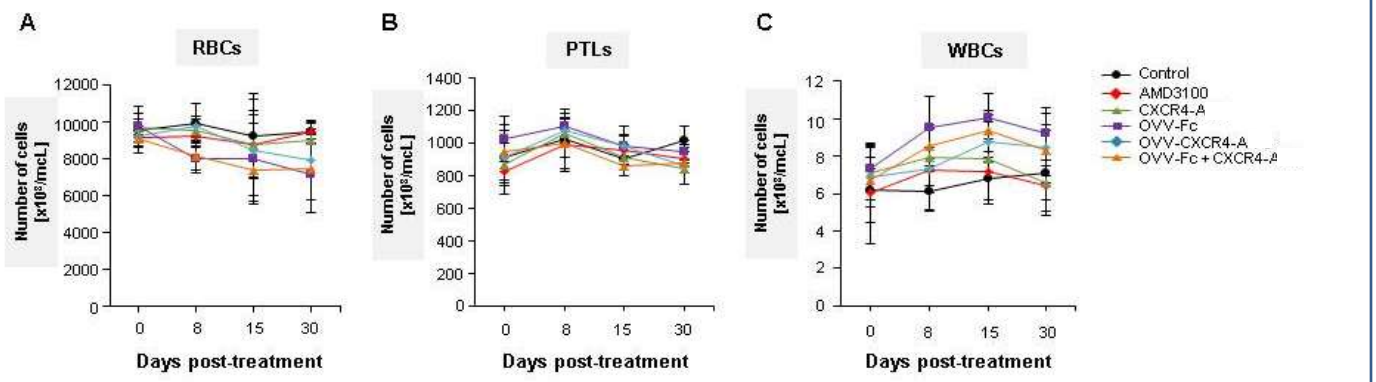

Figure 2. The effect of oncolytic vaccinia virus delivered alone or in combination with soluble CXCR4 antagonists on numbers of red blood cells (RBCs), platelets (PLTs) and WBCs. Mice ($n = 5$) were bled from the retro-orbital sinus to obtain complete CBCs before treatment and on days 8, 15 and 30 after treatment initiation. The numbers of RBCs (**A**), PLTs (**B**) and WBCs (**C**) in the heparinized blood samples were determined using IDEXX ProCyte Dx Hematology analyzer.

- **Inhibition of tumor-immunosuppressive networks and induction of antitumor CD8$^+$ T cell responses by OVV-CXCR4-A-Fc**

The phenotypic analysis of tumor stromal cells and immune infiltrates was performed on single-cell suspensions prepared from peritoneal fluids collected at the time the control mice developed abdominal swelling. Figures 3A-C show that the OVV-CXCR4-A therapy increased total number of tumor-infiltrating CD45$^+$ leukocytes and reduced intratumoral accumulation of immunosuppressive mediators such as CD11b$^+$Ly6C^{low}Ly6G^{high} G-MDSCs compared to the untreated, sCXCR4-A-treated, and OVV-treated counterparts (Fig. 3B; $P = 0.03$ and $P = 0.006$, respectively), and inhibited accumulation of CD4$^+$CD25$^+$Foxp3$^+$ Tregs (Figure 3C; $P < 0.05$). The inhibition of the immunosuppressive network within the TME contributed to increased accumulation of CD8$^+$ TILs, which were detected after sCXCR4-A delivery ($P = 0.02$), and increased by over 3-fold after OVV or OVV-CXCR4-A treatment (Figure 3D; $P < 0.01$). The virotherapy-expanded CD8$^+$ TILs consisted mostly of antigen-experienced

(CD44^{hi}CD62L^{+} and CD44^{hi}CD62L^{-}) cells with less than 5% naïve (CD44^{lo}CD62L^{+}) and double-negative cells (Figure 3E), which was in contrast to the predominantly naïve phenotype of CD8^{+} TILs in the untreated mice. Treatment with sCXCR4-A increased frequencies of CD44^{hi}CD62L^{+} and CD44^{hi}CD62L^{-} CD8^{+} cells compared to control mice though the changes were not significant. The increased percentages of CD8^{+} TILs after oncovirotherapy treatment were associated with higher infiltration of tumor-associated macrophages (TAMs) and DCs, profiled within the CD45^{+} compartment using multi-color flow cytometry and progressive gating strategy (14).

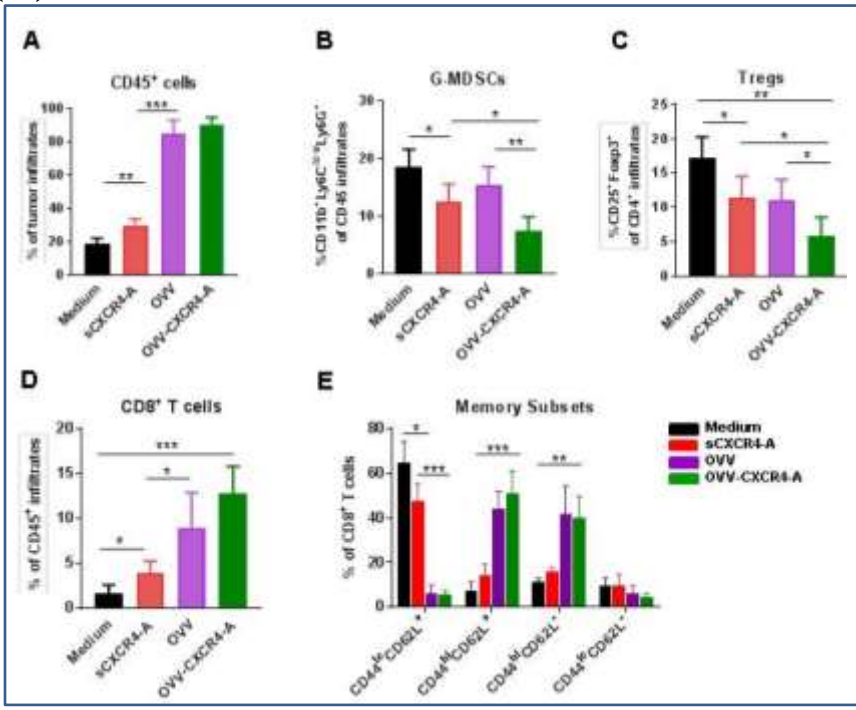

Figure 3. Increased inflammation and inhibition of the immunosuppressive network in the TME by virally-delivered CXCR4-A. (**A-D**) Accumulation of leukocytes (CD45^{+}), G-MDSCs (CD11b^{+}Ly6G^{hi}Ly6C^{lo}), Tregs (CD4^{+}CD25^{+} Foxp3^{+}) and CD8^{+} T cells in peritoneal washes of ID8-T ovarian tumor-bearing mice were analyzed by flow cytometry 8 days after treatments. (**E**) Memory subsets of CD8^{+} T cells were analyzed with mAbs specific for CD44 and CD62L antigens. Background staining was assessed using isotype control antibodies. Data are mean ± SD of three or four independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Staining of the F4/80^{hi}CD24^{lo} cells with CD11b and CD11c showed that after virotherapy treatment, the majority of macrophages exhibited the CD11b^{hi}CD11c^{lo} phenotype, captured by TAM1 subset of macrophages (14) with only small proportions being double-positive for both antigens (CD11c^{hi}CD11b^{hi}) in all treatment groups (Fig. 4A,B). The results presented as the percentages of TAMs within CD45^{+} cells also revealed that the relative proportions of F4/80^{+}CD11b^{hi}CD11c^{lo} cells (TAM1) were higher in all treatment groups compared to control mice (Figure 4A; P < 0.05), whereas no significant differences were observed in the proportions of F4/80^{+}CD11c^{hi}CD11b^{hi} cells (Figure 4B). This was in contrast to increased percentages of CD11b^{+} and CD103^{+} DCs within the F4/80^{lo}CD24^{hi} population after virotherapy treatments (Fig. 4C,D; P < 0.05) with higher numbers of CD103^{+} DCs in OVV-CXCR4-A-treated tumors compared to OVV-treated counterparts (P = 0.04).

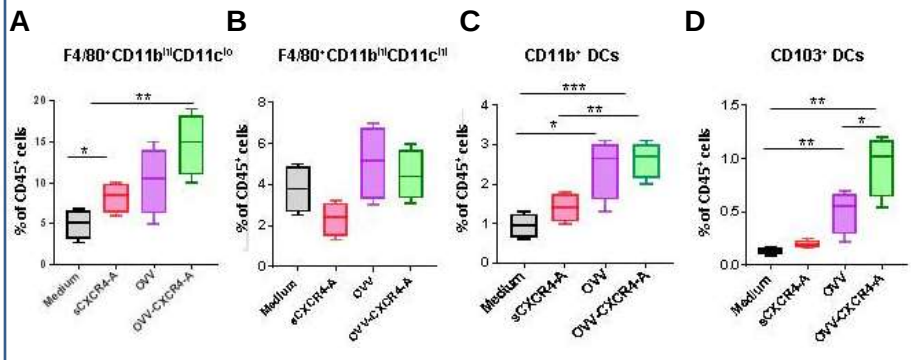

Figure 4. Intratumoral infiltration of CD103^{+} DCs after i.p. treatments with soluble or virally-delivered CXCR4-A. Relative proportions of tumor infiltrating F4/80^{+} CD11b^{hi}CD11c^{lo} (**A**), F4/80^{+}CD11b^{hi} CD11c^{hi} (**B**), CD11b^{+} DCs (**C**), and CD103^{+} DCs (**D**) are depicted as percentages of total CD45^{+} cells. Results are presented as mean ± SD from three or four independent experiments. *P < 0.05, **P < 0.01,***P < 0.001.

<u>Body (from Original SOW)</u>: Specific Aim 2. We analyzed the effect of soluble versus OVV-delivered CXCR4 antagonist on levels and duration of DC vaccine-induced immune responses in tumor-bearing mice.

- **The effect of sCXCR4-A protein and OVV-CXCR4-A on a therapeutic DC vaccine.**

To analyze the effect of soluble versus OVV-delivered CXCR4 antagonist on the level and duration of WT1 peptide-coated DC vaccine-induced immune responses, ID8-T-tumor-bearing mice were treated with sCXCR4-A (10 μg/injection) for 7 days or received one injection of OVV-CXCR4-A (10^8 PFU) before vaccination. Control mice were untreated or treated with the control virus before immunization. The DC vaccine was prepared as previously described (15). The WT1 peptide consisted of an immunodominant polytope (aa 175 to aa 202; CRYGPFGPPSQASS GOARMFPNAPYL) containing H2-IA^b- and H2-D^b-restricted CRYGPFGPPPSQAS and RMFPNAPYL epitopes, respectively. For immunization, we used BM-derived DCs that were cultured in medium supplemented with 10 ng/ml GM-CSF at 37°C for 6 days. DCs were pulsed for 5 h with 10 μg/ml of WT1 peptide, incubated with LPS (1.0 μg/ml) for 1 h to induce maturation, washed, and injected i.v. at concentration of 2×10^6 cells into tumor-bearing mice on day 8 after treatments with the sCXCR4-A protein or the armed virus.

We found that the WT1-coated DC vaccines delivered to control tumor-bearing mice or animals treated with sCXCR4-A had no significant effect on the inhibition of tumor growth compared to the unimmunized counterparts (Fig. 5A and B, respectively). Treatment with OVV significantly reduced tumor growth compared to the vaccine delivered to control mice (Fig. 5C; $P = 0.004$). The highest efficacy was achieved using the armed OVV-CXCR4-A virus prior to vaccination, suggesting that release of the antagonist from virally-infected tumor cells into the tumor stroma along with the virally-mediated reduction of tumor load increased efficacy of the combined treatment compared to that achieved with the control virus (Fig. 5D). The treatment resulted in approximately 5-wk dormancy, which pointed to the ability of the combined CXCR4-A-armed virus and WT1-coated DC vaccine strategy to promote the generation of protective antitumor immune responses.

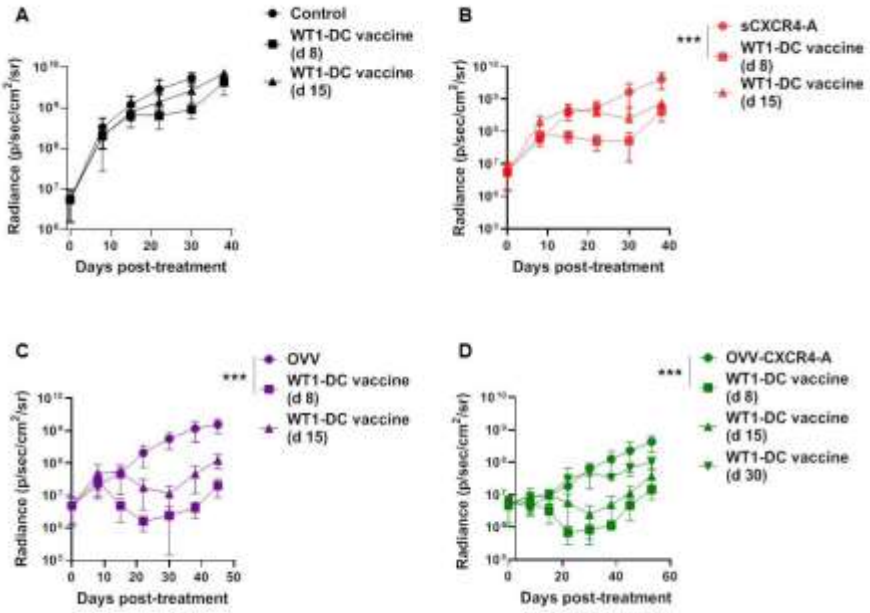

Figure 5. The effect of the CXCR4 antagonist delivered to ID8-T tumor-bearing mice as a soluble protein or expressed by OVV-CXCR4-A on the efficacy of WT1peptide-coated BM-derived DC vaccines. C57BL/6 female mice ($n = 5$ per group) were challenged i.p. with $3x10^5$ ID8-T tumor cells. Control mice were treated with RPMI-1640 medium (**A**). The tumor-bearing mice were treated with sCXCR4-A protein (10 μg/injection for 7 days) (B), OVV (**C**), or OVV-CXCR4-A (10^8 PFU) (**D**) injected i.p. 10 days after tumor challenge. The WT-coated and LPS-matured DCs were injected i.v. (2×10^6 cells) to tumor bearing mice 8, 15 or 30 days after treatments. Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. ***$P < 0.001$.

- **The kinetics of WT1-coated DC vaccine CXCR4 antagonist-treated tumor-bearing mice.**

Based on the kinetics of tumor growth in the control and sCXCR4-A protein-treated mice, the WT1-coated DC vaccines could be delivered only on days 8 and 15 post-treatments. Also, because of an extensive tumor burden the vaccines had no significant effect on inhibition of tumor growth (Fig. 5A,B). This precluded analyses of $CD8^+$

T cell responses after 3 and 4 weeks because the numbers of $CD8^+$ TILs in these mice were less than 10% of total numbers of $CD45^+$ leukocytes (Fig. 3). Vaccinations of tumor-bearing mice 8 and 15 days after virotherapy with the control virus significantly reduced tumor growth compared to vaccines delivered to the untreated mice and their antitumor effects increased when delivered after the OVV-CXCR4-A treatment (Fig. 5C,D; $P < 0.001$). However, the tumor volumes in OVV-treated mice 4 weeks after viral delivery precluded further immunization, and the same treatment delivered to OVV-CXCR4-A-treated mice was largely ineffective (Fig. 5C and D, respectively). Analyses of immune responses revealed increased percentages of $CD8^+$ TILs, with a predominantly effector/effector memory phenotype, in tumor-bearing mice that were vaccinated 8 days after the virotherapy treatment (Fig. 6; $P \leq 0.05$). The percentages of $CD8^+$ TILs decreased when the vaccines were delivered at later time-points, consistent with reductions of inflammatory responses. Despite higher percentages of $CD8^+$ TILs and inhibition of tumor growth after the armed-oncolytic virotherapy- and WT1-coated DC vaccine treatment, the WT1 $tetramer^+$ TILs were at background levels. In a separate experiment, we determined that soluble CXCR4 antagonist delivered to tumor-free mice one day before immunization with WT1-DC vaccine did not interfere with the induction of WT-specific T cells (not shown).

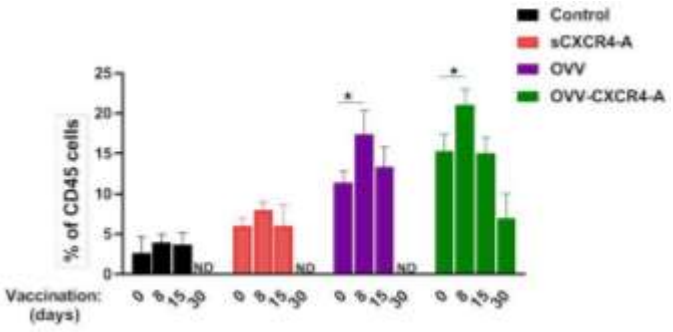

Figure 6. Generation of $CD8^+$ TILs in ID8-T tumor bearing mice by WT1-coated DC vaccines delivered at different time points after treatment with sCXCR4-A protein or OVV-CXCR4-A. ID8-T-tumor-bearing mice ($n = 3$) were treated with sCXCR4-A-Fc (10 μg/injection) for 7 days or received one injection of OVV-CXCR4-A (10^8 PFU) before vaccination. Control mice were untreated or treated with OVV before immunization. WT1 peptide-coated DCs were injected i.v. at the indicated time points, and analyses of $CD8^+$ TILs were performed 8 days after vaccination. Data points represent mean ± SD. *$P \leq 0.05$. ND; not determined.

- **The effect of OVV-CXCR4-A on *in situ* WT1 vaccines**

The relatively low efficacy of the WT1-coated DC vaccine together with the recent findings that intratumoral $CD103^+$ DCs play an important role in stimulating $CD8^+$ TILs and driving immune responses against cancer (16), prompted us to investigate whether $CD103^+$ DCs accumulated in the TME after the CXCR4-A virotherapy treatment would be more efficacious, when combined with an adjuvanted WT1 peptide, than the BM-derived counterparts. Given the profound association of intratumoral stimulatory DCs with patient outcome (17) and thigher infiltration of $CD103^+$ DCs after OVV-CXCR4 treatment, we explored whether expansion of $CD103^+$ DCs in the TME by local injection of FLT3L (referred to hereafter as FL) cytokine (18) would enhance therapeutic efficacy of the combined treatment by promoting tumor antigen presentation and priming of T cells following virotherapy-mediated immunogenic cell death (19). The WT1 peptide containing H2-IA^b-restricted CRYGPFGPPPSQAS and H2-D^b-restricted RMFPNAPYL epitopes (8, 13) was injected i.p. in combination with polyI:C (50 μg/injection) to ID8-T-bearing mice (50 μg/injection) 2 days after FL delivery (Fig. 7A). Additional groups of tumor-bearing mice received only virotherapy treatments before immunization to determine the importance of FL-expanded $CD103^+$ DCs in the induction of tumor-antigen-specific T cells and inhibition of tumor growth. As shown in Fig. 7B, vaccination of mice after OVV and FL treatment combination exhibited potent antitumor activities extending the survival by ~20 days compared with mice treated with the virus or virus and WT1 vaccine only ($P < 0.001$). This regimen also elicited measurable WT1-specific $CD8^+$ T cell responses compared to those induced by vaccination without prior FL treatment ($P = 0.03$; Fig. 7C,D). The CXCR4-A-armed virus followed by FL-mediated expansion of $CD103^+$ DCs prior to vaccination was most effective in inhibiting tumor growth (median survival of 69 days; Fig. 7E) and inducing WT1-2D^b/RMFPNAPYL $tetramer^+CD8^+$ TILs (Fig. 7F,G), which could reflect the reduction of immunosuppressive elements in the TME by the CXCR4 antagonist. Importantly, the immunization regimen enhanced the percentages of WT1 $tetramer^+CD8^+$ TILs and was associated with tumor-free survival in approximately 10% of treated mice.

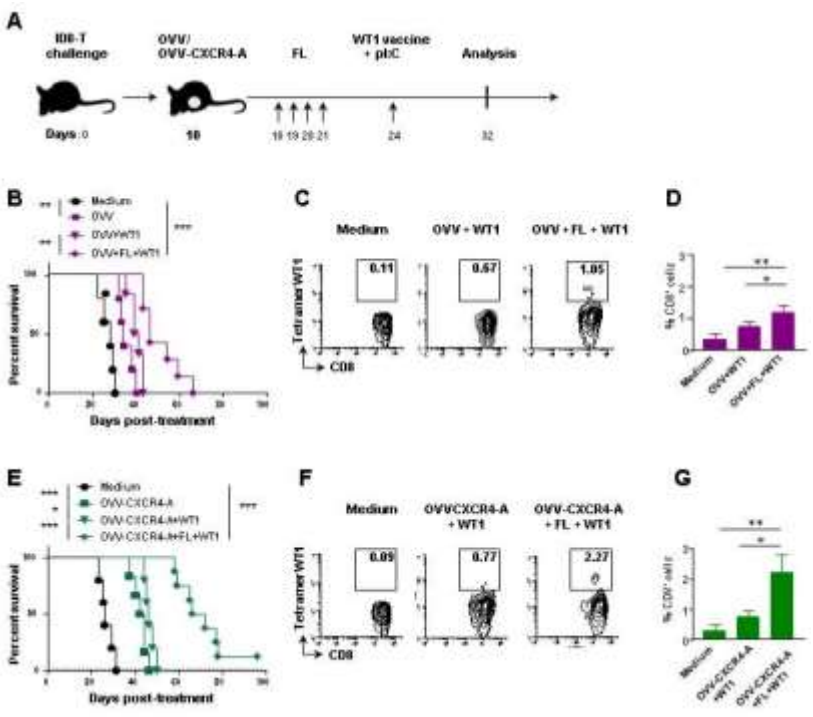

Figure 7. An adjuvanted WT1 vaccine delivered after oncovirotherapy and FL treatment generates WT1-specific $CD8^+$ TILs. (**A**) Graphical time-line of the treatment. (**B**) Survival after WT1 immunization of OVV- and OVV plus FL-treated mice. Kaplan-Meier survival plots were prepared and significance was determined using the log-rank method. $^{**}P < 0.01$. Representative staining (**C**) and relative proportions of WT1 $tetramer^+CD8^+$ TILs (**D**) after the combined treatment of OVV and WT1 vaccine as well as OVV and FL treatment followed by WT1 vaccination are shown. (**E**) Survival of tumor-bearing mice after WT1 immunization of OVV-CXCR4-A and OVV-CXCR4-A plus FL-treated mice. $^{*}P < 0.05$, $^{***}P < 0.001$. Representative staining (**F**) and relative proportions of WT1 $tetramer^+CD8^+$ TILs (**G**) after the combined treatment of OVV-CXCR4-A and WT1 vaccine and OVV-CXCR4-A plus FL treatment followed by WT1 vaccination are shown.

<u>Body (from Original SOW)</u>: Specific Aim 3. We examined the mechanisms associated with migration of tumor-specific T cells to tumor sites after adoptive T cell transfer (ACT).

- **The effect of the CXCR4 antagonist on migration of WT1-$tetramer^+$ $CD8^+$ T cells to tumor sites and inhibition of tumor progression.**

We performed ACT of vaccine-generated T cells to ID8-T-bearing recipient mice that are treated with OVV-CXCR4-A. The control mice are treated with PBS or OVV only. Because molecular mechanisms governing T cell trafficking to tumors stem from observations at nonmalignant sites, where extravasation depends on stepwise adhesive interactions within the blood vessels, we first evaluated whether treatment with the CXCR4-A-armed virus enhances migration of adoptively transfer $CD8^+$ T cells. We also examined the potential mechanisms by which OVV-CXCR4-A promotes T cell accumulation by transcriptome analysis of pathways involved in T cell recruitment, trafficking and retention. Experiments using the sCXCR4-A protein prior to the adoptive transfer were not included in the analysis because the soluble antagonist did not show significant effect on tumor growth and changes in the TME.

The $CD8^+$ T cells were isolated from the spleen of ID8-T tumor-bearing mice 30 days after treatment with OVV-CXCR4-A by negative selection using MACS Miltenyl Biotech kit and delivered i.v. to untreated tumor-bearing recipients (control) or animals treated with OVV or OVV-CXCR4-A 24 h after the treatment. The omental tumors were resected 2 h later and analyzed by flow cytometry and transcriptome analysis for infiltration of $CD8^+$ T cells and pathways involved in T cell trafficking, respectively. We found that OVV-CXCR4-A promotes increased intratumoral $CD8^+$ T cell accumulation, compared with control mice or OVV-treated animals (Fig. 8). The transcriptome analysis revealed that, compared with controls, OVV-CXCR4-A treated ID8-T tumors had significant upregulation of T cell chemotaxis/migration pathways (Fig. 9A,B), such as transcripts involved in primary tethering and rolling of naïve and T_{CM} cells (e.g. ITGB7 binding of MAdCAM-1) and sialomucin-like endothelial molecules (e.g. GlyCAM1, VCAM1, ICAM1) mediating T cell extravasation.

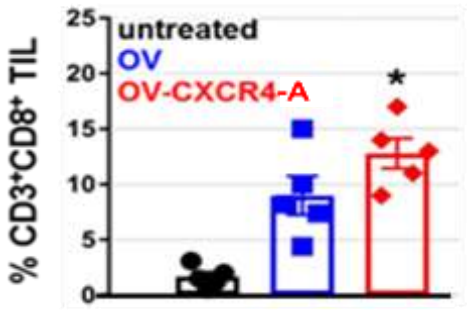

Figure 8. Elevated numbers of TILs in ID8-T tumors treated with OVV-CXCR4-A observed by flow cytometry 2 h after adoptively-transferred $CD8^+$ splenocytes. The flow cytometry analysis was carried out on single cell suspensions prepared from omental tumors and gated on $CD45^+$ cells.

Upregulation of chemokines and chemokine receptors that are critical for T cell mobilization and retention further demonstrated profound effects of OVV-CXCR4-A on ovarian tumor endothelium to facilitate efficient T cell trafficking to the TME (Fig. 9C).

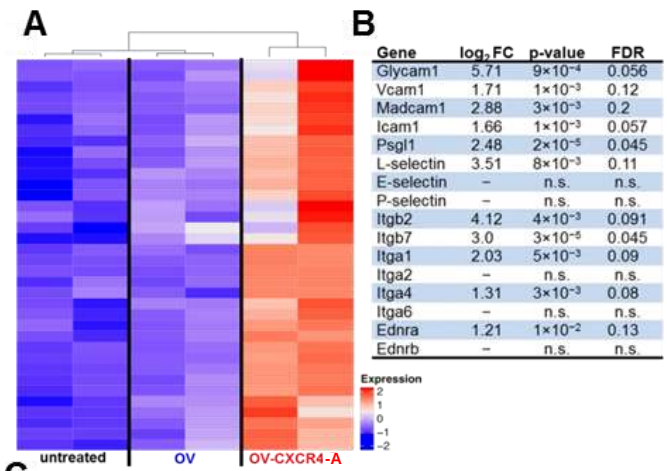

Figure 9. Transcriptome analysis of changes in pathways involved in T cell trafficking mediated by OVV-CXCR4-A treatment. The omental tumors were resected 24 h after oncolytic virotherapy treatment and subjected to RNAseq. (**A**) RNAseq of tumors revealed unique T cell trafficking signature. (**B**) Fold change of select genes critical for trafficking and adhesion. (**C**) Cognate chemokine and chemokine receptor expression changes.

Next, we examined efficacy of the combined treatment of OVV-CXCR4-A virotherapy and ACT in ID8-T tumor-bearing mice. For the adoptive transfer, $CD8^+$ T cell are isolated from spleens of tumor bearing mice treated with OVV-CXCR4-A, combined with WT1 peptide-coated DCs (20:1 ratio) to induce proliferation of T_{EFF} cells and mount a secondary response (15). After 3 days of stimulation, T cells (10^7 cells/injection) were injected i.v. to tumor-bearing mice 8 and 15 days after OVV-CXCR4-A virotherapy treatment. Progression of tumor growth, quantified by bioluminescence imaging, revealed that the combination of OVV-CXCR4-A and ACT delivered on day 8 after the virotherapy treatment was most effective in inhibition of tumor growth and metastatic dissemination, extending the slower rate of tumor progression and survival for approximately 8 and 5 weeks compared to the same treatments in control mice or OVV-CXCR4-A-treated counterparts, respectively (Fig. 10A,B). On the other hand, the adoptive transfer of T cells on day 15 after was less effective in inhibiting tumor growth resulting in less than 5 weeks dormancy period, suggesting an important role of the tumor load and TME in supporting efficacy of adoptively transferred T cells.

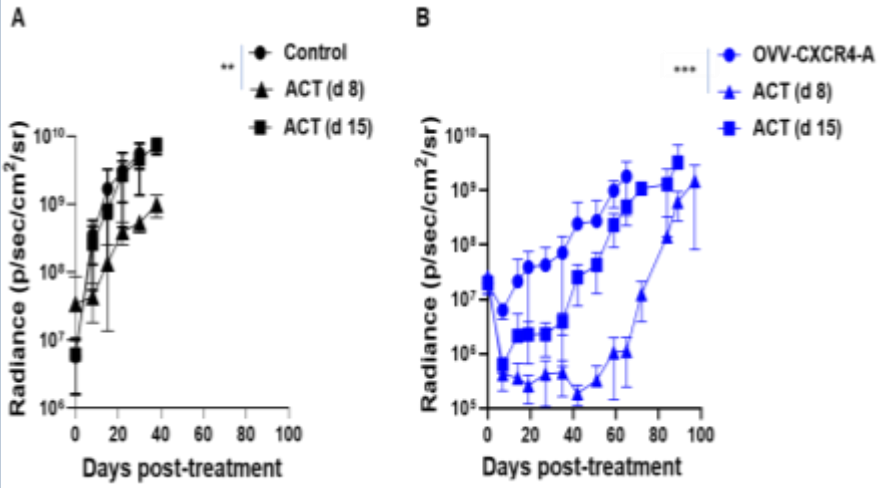

Figure 10. The effect of OVV-CXCR4 and ACT on inhibition of tumor growth. C57BL/6 mice (n = 5 per group) were challenged i.p. with 3x10^5 ID8-T cells. Control mice were treated with RPMI-1640 medium (**A**) or with OVV-CXCR4-A (**B**) injected i.p. 10 days after tumor challenge. The ACT was delivered i.v. (10^7 cells/injection) to tumor bearing mice 8 and 15 days after treatments. Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

- **OVV-CXCR4-A promotes CD8$^+$ T cell accumulation and overcomes limited CD8$^+$ T$_{eff}$ trafficking across ovarian tumor vasculature as visualized by real-time intravital microscopy (IVM).**

We have previously shown that OVV-CXCR4-A improves ID8-T tumor control by increasing CD8$^+$ TIL frequency compared with OVV control. To evaluate mechanisms associated with this phenotype (Fig. 11A,B), we assessed the TME for changes in TIL localization relative to vasculature. A distance score measurement revealed closer relative proximity of TILs to vessels in OVV-CXCR4-A treated tumors compared to control (Fig. 11C) and a greater number of TILs within 100 μm of vessels (Fig. 11D).

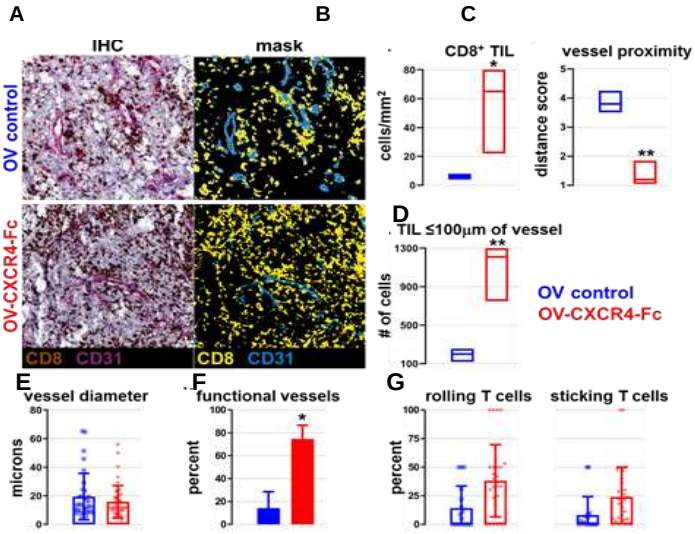

Figure 11. OVV-CXCR4-A treatment promotes increased CD8$^+$ TIL via alreration in T cell trafficking evaluated by IVM. (**A**) Representative CD8 and CD31 IHC staining in ID8-T tumors treated with OVV-CXCR4-A. Image analysis of CD8$^+$ and CD31$^+$ cells used perform proximity analyses. (**B**) CD8$^+$ TIL frequency. (**C**) Proximity of CD8$^+$ cells to CD31$^+$ cells. (**D**) Number of CD8$^+$ cells within 100 μm of CD31$^+$ vessels. (**E**) Quantification of vessel diameter. (**F**) Frequency of functional vessels/field assessed by the presence of dynamic flow. (**G**) Proportion of activated CD8$^+$ T$_{eff}$ cells exhibiting rolling or sticking to vasculature of treated ID8-T tumor-bearing mice. $^*P < 0.05$; $^{**}P < 0.01$.

We next evaluated the possibility that OVV-CXCR4-A promotes T cell trafficking via direct effects on tumor vasculature and/or effects on factors critical for T cell chemotaxis. This is an important question because the precise mechanisms that govern delivery of T cells across tumor "vascular checkpoints" are not known. While IHC is the clinical standard for defining tumor characteristics including vascularity, it provides only a static 'snapshot' (Fig. 11A) that may not reflect active processes occurring *in situ*. To examine the ability of tumor vasculature to support lymphocyte trafficking, we performed IVM of labeled tumor-specific T cell trafficking into the ovarian TME. IVM measurement of tumor vasculature showed no difference in vessel diameter by OVV-CXCR4-A treatment (Fig. 11E) that could account for enhanced infiltration; however, a greater proportion of

vessels was functional compared to control as defined by presence of adequate blood flow (Fig. 11F). Adoptive transfer of labeled tumor-specific T cells revealed OVV-CXCR4-A also enhanced the proportion of $CD8^+$ T_{eff} exhibiting rolling or sticking to tumor vascular endothelium (Fig. 11G). These findings reveal the profound ability of OVV-CXCR4-A to reprogram the TME - not only by decreasing immunosuppressive networks, but also modifying critical components of T cell trafficking and retention.

In summary, the results of flow cytometry and RNAseq analyses revealed the ability of OVV-CXCR4-A to reprogram the TME by decreasing the immunosuppressive network and modifying critical components of T cell trafficking and retention.

- **Key Research Accomplishments**:

1) We demonstrated that the more efficacious inhibition of ovarian tumor growth by i.p. delivery of the CXCR4 antagonist, either by the oncolytic vaccinia virus or in a soluble form, was associated with higher concentrations of sCXCR4-A in the tumor compared with systemic delivery. The i.p. treatment also resulted in background levels of the antagonist in sera or other organs, which contrasted with ~2-fold higher levels of sCXCR4-A in systemic tissues after i.v. delivery.

2) We showed that i.p. delivery of the 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 reduced immunosuppressive network in the TME and increased infiltration of $CD8^+$ T lymphocytes.

3) We demonstrated that the immunogenic cell death-inducing armed-oncolytic virotherapy has been able to act as *in situ* vaccines in ovarian tumor-bearing syngeneic mice and generate antitumor immune responses that inhibited progression of tumor growth.

4) The virally-mediated *in situ* vaccination could be boosted with an adjuvanted WT1-specific peptide-based vaccine after expansion of $CD103^+$ DC population at the tumor site.

5) The transcriptomic analysis of pathways involved in T cell recruitment, trafficking and retention revealed that, compared with controls, OVV-CXCR4-A treated ID8-T tumors had significant upregulation of T cell chemotaxis/migration pathways, such as transcripts involved in primary tethering and rolling of T cells (e.g. ITGB7 binding of MAdCAM-1) and sialomucin-like endothelial molecules (e.g. GlyCAM1, VCAM1, ICAM1) mediating T cell extravasation.

6) The transcriptome analysis, flow cytometry, and confocal microscopy revealed increased infiltration of adoptively transferred $CD8^+$ T cells, isolated from splenocytes of ID8-T-challenged mice after treatment with the armed oncolytic virotherapy and *in vitro* stimulation with WT1 peptide-coated DCs, to omental tumors compared to untreated controls or animals treated with the control virus.

Conclusions: The CXCR4 antagonist-armed oncolytic vaccinia virus inhibits the immunosuppressive network at the tumor site and provides target antigens as well as danger signals for induction of adaptive immune responses. By inducing immunogenic tumor cell death and antigen release at the tumor site, the virally-delivered vaccination is personalized and can be boosted with tumor antigen peptide-based vaccines or ACT. Our findings reveal a profound ability of the OVV-CXCR4-A treatment to reprogram the TME and facilitate infiltration of $CD8^+$ TILs, thus representing a potent therapy for ovarian CICs with a broad antitumor repertoire.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report.

- **How were these results disseminated to communities of interest?**

Nothing to Report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Nothing to Report.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

While most women with advanced stage OC initially respond to surgery and first-line chemotherapy, more than 70% of patients eventually die of recurrent disease within 5 years of diagnosis. Our group reported that OC patients with higher frequencies of intraepithelial $CD8^+$ TILs demonstrated improved survival compared with patients with lower frequencies (4). Subsequently, a meta-analysis of ten studies with 1,815 OC patients confirmed the original observation (20). These studies support immunotherapy strategies generating large pools of tumor-specific T cells for OC treatment. However, although tumor-specific T cells were generated by vaccine trials targeting the *bona-fide* tumor antigen such as NY-ESO-1 (7, 21), long-term tumor control was limited due to (i) insufficient expansion and persistence of T cells specific for tumor antigens (epitope spreading) and (ii) inability to overcome OC inhibitory networks. While $CD8^+$ T cell localization in tumors is widely recognized as an essential determinant of tumor immunity, surprisingly little is known about the mechanisms driving T cell trafficking across OC vasculature and even less information is available about the mechanisms that regulate abundance of intratumoral stimulatory DCs in the TME and their influence on antitumor responses of TILs, frequencies of exhausted T cells, patient responsiveness to anti-PD-1 immunotherapy and overall survival.

In this study, we have focused on the CXCR4/CXCL12 axis because of its multiple adverse effects on OC progression (22, 23). These include cancer cell migration, invasion, stimulation of angiogenesis (24) as well as intra-tumoral recruitment of endothelial progenitor cells (25, 26), suppressive myeloid cells (27-29), and Tregs (29, 30). Therefore, modulation of the CXCL12/CXCR4 axis in OC could impact multiple aspects of OC pathogenesis including innate and adaptive immune mechanisms of tumor destruction. Because innate-resistance of non-responding tumors together with immunosuppressive TME negatively affect immunotherapy treatments, the priming with CXCR4 antagonist-armed virotherapy followed by tumor-specific peptide-vaccines or ACT boost strategies could reduce tumor load and drove effective antitumor immunity in OC patients.

- **What was the impact on other disciplines?**

Nothing to Report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

The described immunization strategy may help in designing new vaccine approaches against ovarian cancer.

5. CHANGES/PROBLEMS:

Nothing to report

6. PRODUCTS:

Publications:

Mistarz, A., Komorowski, M.P., Graczyk, M.A., Gil, M., Jiang, A., Opyrchal, M., Rokita, H., Odunsi, K.O., and **Kozbor, D.** Recruitment of Intratumoral $CD103^+$ Dendritic Cells by a CXCR4 Antagonist-armed Virotherapy Enhances Antitumor Immunity. Mol.Therapy-Oncolytics, 14: 233-245. 2019.

Presentations:

Oct. 23, 2017 Invited Speaker: 20th Annual Upstate New York Immunology Conference. The Sagamore Resort and Conference Center, Bolton Landing, NY: "Targeting Drug-resistant Ovarian Cancer with Oncoimmunotherapy".

Jan. 24, 2019 Invited speaker: Tumor Immunology & Immunotherapy retreat 2019
Holiday Valley Road, Ellicottville, NY: "Expansion of intratumoral dendritic cells after armed oncovirotherapy augments efficacy of a therapeutic cancer vaccine".

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Danuta Kozbor, Ph.D.
PI
Effort: 2.4 calendar months (no change)

Adekunle Odunsi, M.D., Ph.D.
Co-Investigator
Effort: 0.24 calendar months (no change)

Li Yan, Ph.D.
Biostatistician
Effort: 0.36 calendar months

Anna Mistarz, M.Sc.
Research Affiliate
Effort: 12.0 calendar months

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report.

- **What other organizations were involved as partners?**

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Not applicable

- **QUAD CHARTS:** Not applicable

9. APPENDICES:

- Other Support

- Updated Curriculum Vitae

- Mistarz, A., Komorowski, M.P., Graczyk, M.A., Gil, M., Jiang, A., Opyrchal, M., Rokita, H., Odunsi, K.O., and **Kozbor, D.** Recruitment of Intratumoral $CD103^{+}$ Dendritic Cells by a CXCR4 Antagonist-armed Virotherapy Enhances Antitumor Immunity. Mol.Therapy-Oncolytics, 14: 233-245. 2019.

OTHER SUPPORT
KOZBOR, DANUTA

CURRENT/ACTIVE

U01CA233085 (PIs: Odunsi/Kozbor/Gambotto) 9/23/19-8/31/24
NIH/NCI
Funding level:
Time Commitment: 2.44 calendar
Title: Reprogramming T cells and the tumor microenvironment to overcome multiple primary and acquired immune resistance mechanisms in ovarian cancer

Project Goals/aims: The overall objective of this study is to reprogram the ovarian tumor microenvironment with an "armed" oncolytic vaccinia virus delivering CXCR4 antagonist, PD1, and LAG3 inhibitors, while minimizing the potential for high rates of systemic toxicities.
Overlap: None

PENDING

P50CA159981-07A1 (PIs: Odunsi/Moysich) 4/1/21-3/31/26
NIH/NCI
Funding level:
Time Commitment: 1.2 CM (Project 1)
Title: Roswell Park Ovarian Cancer SPORE
Project Goals/aims: The overall objective of this project is to elucidate the mechanisms that contribute to tumor progression or protection in patients with chemo-resistant ovarian cancer in the face of a potent combinatorial immunotherapy approach.
Overlap: None

ACTIVE TO COMPLETED

(This award)
W81XWH16-1-0146 (PI: Kozbor) 6/15/16-6/14/20
Department of Defense (OC150418)
Investigator-Initiated Research Award grant
Funding Level: (NCE)
Time commitments: 2.4 calendar
Title: CXCR4 antagonist as an adjuvant in immunotherapy of epithelial ovarian cancer
Project Goals/aims This application proposed to test the hypothesis that differences in the level of CXCR4 antagonists in the tumor and lymphoid/non-lymphoid tissues after the targeted versus soluble form of delivery will affect the treatment efficacy by modulating the recruitment and survival of immune cells in tumor sites.

R03CA223623 (PI: Kozbor) 7/16/18-6/30/20
NIH/NCI
Funding Level:
Time commitments: 1.2 calendar
Title: The role of CREB3L1 in synergy between oncolytic vaccinia virus and doxorubicin
Project Goals/aims: This study examined expression of the membrane-bound transcription factor CREB3L1 in clinical specimens of ovarian tumor to explore its association with doxorubicin resistance and involvement in vaccinia virus-mediated increases in sensitivity. The insight obtained from understanding the mechanisms associated with the synergistic interaction between oncolytic virotherapy and doxorubicin may predict which patient responds to doxorubicin and if these two therapeutic agents could be combined to enhance survival and quality of life.

P30 CA016056 Johnson (PI) 5/1/18-4/30/19
NIH Cancer Center Support Grant
National Cancer Institute
“ Developmental funds for reprogramming anti-tumor immunity with oncolytic viruses”
Kozbor (PI)

Project Goals/ aims: The overall goal of this study was to prevent cancer recurrence and metastasis by eradicating CD44 antigen-expressing cancer stem cells and simultaneously target the tumor microenvironment promoting tumor growth. Using a panel of established human ovarian cancer cell lines, the aim is to investigate the plausible rationale for how CD44 increases susceptibility of cancer cells to vaccinia infection and affects induction of innate immunity.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Danuta Kozbor

eRA COMMONS USER NAME (credential, e.g., agency login): KOZBOR

POSITION TITLE: Professor and Member

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
VII General Ed. Lyceum, Poland	B.Sc	06/1971	Biology
Jagiellonian University, Krakow, Poland	M.Sc	06/1976	Molecular Biology
Karolinska Institute, Stockholm, Sweden		12/1978	Tumor Biology
Queen's University, Kingston, Ontario, Canada	Ph.D.	10/1982	Microbiol/Immunol

A. Personal Statement

The overall goal of our research is to elucidate molecular and cellular mechanisms of innate immune responses induced by oncolytic vaccinia viruses in cancer patients. We design novel approaches to induce antitumor immunity against chemoresistant ovarian tumors by a CXCR4 antagonist-armed viral oncotherapy (US patent number: 9,296,803; Methods and compositions containing Fc fusion proteins for enhancing immune responses; March 29, 2016) alone or in combination with immune checkpoint inhibitors. We are also investigating the effect of CREB3L1 transcription factor and CD44 antigen expression as well as STING signaling in drug-resistant ovarian cancer variants on susceptibility to oncolytic vaccinia virus infection and induction of type I IFN expression. These studies have the potential to be translated into the clinic though collaboration with our clinical investigators and will help in exploring the mechanistic underpinnings of vaccinia virus-immune system interactions. In the past, we have evaluated the effect of selective disruption of vasculature by photodynamic therapy (PDT) on the therapeutic activity of systemically administered oncolytic vaccinia virus expressing an antagonist of the CXCR4 receptor against syngeneic murine tumors and human tumor xenografts.

As part of the CCSG effort, and in collaboration with Dr. Kunle Odunsi, we are analyzing the mechanisms by which oncolytic virotherapy with the CXCR4 antagonist (OVV-CXCR4-A-Fc) and PD1/PDL1 blockade overcome the vascular endothelial barrier to promote intratumoral T cell trafficking and accumulation. The OVV-CXCR4-A-Fc construct has been developed in my laboratory. Toxicology studies are carried out in response to the FDA request of the pending Investigational New Drug (IND) application for the use of OVV-CXCR4-A in clinical trials.

I am also committed to providing quality training in the development of future leaders in scientific and translational research. For the past fifteen years, I have been involved in numerous activities of the Graduate Program within the Department of Immunology at Roswell Park. This includes i) serving on Thesis/Dissertation Committees for Master and PhD students, ii) serving as primary instructor of Immunology Student Seminar series, iii) presenting lectures to graduate students, iv) serving as Chair of Qualifying Exam Committee, v) participating in admissions review process, vi) supervising pre- and post-doctoral trainees, and vii) supervising rotation and summer students.

As a postdoctoral fellow at the Wistar Institute in Philadelphia, PA, I gained experience in molecular genetics during the research on the mechanisms of oncogene activation in human tumors by chromosomal translocation. Subsequently, as an independent investigator, I expanded my research to virology by analyzing the effect of perinatal HIV infection on disease progression, and induction of HIV-specific immune responses using a recombinant vaccinia virus as a vector for AIDS vaccine delivery. As PI or Co-Investigator on several previous NIH- and institute-funded grants, I laid the background for the proposed research by developing oncolytic

vaccinia viruses expressing mimotopes of tumor associated antigens and by targeting the antigens to the activating Fc gamma receptor on dendritic cells. In addition, I successfully administered the projects (e.g. staffing and budget), collaborated with other researchers, and produced several peer-reviewed publications from each project. I am aware of the importance of frequent communication among project members and constructing a realistic research plan, timeline and budget.

B. Positions and Honors

Positions and Employment

1982-1985	Postdoctoral Fellow, National Cancer Institute of Canada, Wistar Institute, Philadelphia, PA
1985-1989	Associate Scientist, The Wistar Institute, Philadelphia, PA
1989-1991	Assistant Professor, Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, PA
1991-1996	Assistant Professor, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA
1996-1998	Associate Professor, Allegheny University of the Health Sciences, Philadelphia, PA
1998-1999	Associate Professor, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA
1999-2002	Professor, Center for Neurovirology and Cancer Biology, Temple University, Philadelphia, PA
2002-present	Associate Professor and Associate Member, Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY
2020-present	Professor of Oncology and Immunology, Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY

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Other Professional Activities and Honors

1980-1982	National Cancer Institute of Canada Studentship
1982-1986	National Cancer Institute of Canada Fellowship
1986-1987	Consultant on the Hybridoma Technology, World Health Organization (WHO Regional Office for Southeast Asia, New Delhi, India)
1986-1988	Consultant on the Hybridoma Technology, Dow Chemicals, Midland, MI
1988-1989	Consultant on the Hybridoma Technology, DuPont Company, Wilmington, DE
1994-1999	Ph.D. Thesis Advisory Committee, University of Pennsylvania, Philadelphia, PA
1996-1999	Clinical Trials in Zambia: "A phase II evaluation of curdlan sulfate bolus infusion alone or in combination with quinine in patients infected with malaria".
1995-2002	Investigator, Philadelphia Pediatric AIDS Clinical Trials Unit
1999-2002	Investigator, Penn Center for AIDS Research, Philadelphia, PA
1999-2006	Editorial Board Member, Clinical & Diagnostic Laboratory Immunology
1999-2007	Reviewer, NIH/NIAID Special Emphasis Review Panel on HIV Vaccine Research & Design NIH-NIAID-RFA-05001 Leadership for HIV/AIDS Clinical Trials Networks: Vaccines.
2000	Visiting Professor, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan2006
2006	Patent: March 14, 2006; "β-Glucans encapsulated in liposomes" (US patent 7,011.845).
2004-2013	Chair, Institute Biosafety Committee, Roswell Park Comprehensive Cancer Center, Buffalo, NY
2012	*Ad Hoc* Reviewer; NIH, Cancer Immunopathology and Immunotherapy Study Section

C. Contribution to Science

1. Cancer Immunotherapy and Vaccines

A major challenge for inducing antitumor immune responses with native or modified tumor/self-Ags in tumor-bearing hosts relates to achieving efficient uptake and processing by dendritic cells (DCs) to activate immune effector cells and limit the generation of immunosuppressive network in the tumor microenvironment. We have demonstrated that immunization of adoptively transferred T cells in tumor-bearing mice with a CD166 cross-reactive mimotope 47-LDA, expressed in the context of the activating Fc fusion protein, induced higher levels of antitumor immune responses and protection than the 47-LDA polypeptide-DC vaccine. The antitumor efficacy of the therapeutic 47-LDA-Fc-DC vaccine was comparable to that achieved by an oncolytic vaccinia virus (OVV) expressing the 47-LDA-Fc fusion protein, paving the way for testing novel anticancer treatments.

Because the CXCR4 receptor for the CXCL12 chemokine is one of the key stimuli involved in signaling interactions between tumor cells and their microenvironment, we have also investigated whether inhibition of this pathway by oncolytic viruses expressing the CXCR4 antagonist increases efficacy over that mediated by oncolysis alone. We are unique in demonstrating that targeting CXCR4 signaling through an oncolytic vaccinia

virus yields a significant therapeutic impact against primary and metastatic breast and ovarian cancers. I served as a senior investigator in all of these studies.

Komorowski, M., McGray, A.J.R., Kolakowska, A., Eng, K., Gil, M., Opyrchal, M., Litwinska, B., Nemeth, M.J., Odunsi, K.O., and **Kozbor, D**. Reprograming antitumor immunity against chemoresistant ovarian cancer by a CXCR4 antagonist-armed viral oncotherapy. Mol. Therapy-Oncolytics, 2016, 3:16034-1648.

Komorowski, M., Tisonczyk, J., Kolakowska, A., Drozdz, and **Kozbor, D.** Modulation of the tumor microenvironment by CXCR4 antagonist-armed viral oncotherapy enhances the antitumor efficacy of dendritic cell vaccines against neuroblastoma in syngeneic mice. Viruses, 2018, 10:455-471.

McGray, A.J.R, Huang, R.Y., Battaglia, S., Eppolito, C., Miliotto, A., Stephenson, K.B., Lugade, A.A., Webster, G., Lichty, B.D., Seshadri, M., **Kozbor, D.,** and Odunsi, K. Oncolytic Maraba virus armed with tumor antigen boosts vaccine priming and reveals diverse therapeutic response patterns when combined with checkpoint blockade in ovarian cancer. J. ImmunoTherapy of Cancer. 2019, 7: 189-2005.

Mistarz, A., Komorowski, M.P., Graczyk, M.A., Gil, M., Jiang, A., Opyrchal, M., Rokita, H., Odunsi, K.O., and **Kozbor, D.** Recruitment of Intratumoral CD103^{+} Dendritic Cells by a CXCR4 antagonist-armed Virotherapy Enhances Antitumor Immunity. Mol.Therapy-Oncolytics, 2019,14: 233-245. 2019.

2. T Cell Activation during Perinatal HIV Infection

The purpose of these studies was to examine the changes in cytokine/chemokine expression and T cell activation during progression towards AIDS in infants born to HIV-infected mothers as well as adult HIV-infected patients. We have demonstrated for the first time a protective role of chemokine against vertical HIV infection, association of early HIV-specific Th1 and CTL responses with slow disease progression, and expansion of Vδ1+T lymphocytes during progression of HIV infection. I served as a senior investigator in all of these studies.

Hyjek, E., Lischner, H. W., Hyslop, T. Bartkowiak, J., Kubin, M., Trinchieri, G., and **Kozbor, D**. Cytokine patterns during progression to AIDS in children with perinatal HIV-1 infection. J. Immunol., 1995, 155:4060-4071.

Hyjek, E. M., Bartkowiak, J., Drozdz, R., Wasik, T. J., Jasinski, M., Lischner, H. W. and **Kozbor, D**. Evidence for B cell-mediated activation of Vδ1^{+} T lymphocytes during progression of HIV infection. J. Immunol. 1997, 158:464-474.

Wasik, T. J., Jagodzinski, P. P., Hyjek, E. M., Lischner, H. W., and **Kozbor, D**. Diminished HIV-specific CTL activity associated with enhanced type 2 responses to HIV-specific peptides during perinatal HIV infection. J. Immunol. 1997, 158:6029-6036.

Wasik, T. J., Lischner, H. W., Jasinski, M., Bratosiewicz, J., Whiteman, V. E., Rutstein, R., Starr, S. E., Douglas, S., Kaufman, D., Sison, A. V., and **Kozbor, D.** Protective role of nonlytic immune responses in vertical HIV transmission. J. Immunol. 1999, 162:4355-4364.

3. Development of HIV Vaccine

In addition to the contribution described above, with a team of collaborators, I was involved in the development of HIV vaccines by identifying immunogenic and conserved epitopes within the HIV Envelope and Gag/Pol antigen for induction of protective cellular responses. I served as a senior investigator in all of these studies.

Kmieciak, D., Jasinski, M., Teppler, H., Pientka, J., Hsu, S. H., Takahashi, H., Okumura, K., Kaneko, Y., and **Kozbor, D**. The effect of deletion of the V3 loop of gp120 on induction of cytotoxic T cell responses and HIV gp120-mediated pathogenesis. J. Immunol. 160:5676-5683, 1998.

Kmieciak, D., Bednarek, I., Takiguchi. M., Bratosiewicz, J., Wierzbicki, A., Wasik, T., Teppler, H., Pientka, J., Hsu, S. H., and **Kozbor, D**. The effect of epitope variation on the profile of CTL responses to the HIV envelope glycoprotein. International Immunol. 10:1789-1799, 1998.

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4. Mechanisms of Oncogene Activation in Human Malignancies

I was involved in characterizing mechanisms of oncogene activation (abl and myc) by chromosomal translocation in leukemia and solid tumors.

Kozbor, D., Finan, J., Nowell, P.C., and Croce, C.M. The gene encoding the T4 antigen maps to human chromosome 12. J. Immunol., 136: 1141-1143, 1986.

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5. Production of Human Monoclonal Antibodies

Development of the hybridoma technique has revolutionized treatments of autoimmunity and cancer. I have pioneered the EBV-hybridoma technology for production of human monoclonal antibodies.

Kozbor, D. and Roder, J.C. Requirements for the establishment of high-titered human monoclonal antibodies against tetanus toxoid using the Epstein-Barr virus technique. J. Immunol. 127: 1275-1280, 1981.

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D. Research Support

Ongoing Research Support

U01 CA233085 (PIs: Odunsi/Kozbor Gambotto) 9/1/19-8/31/24
NIH/NCI
"Reprograming the Tumor Microenvironment to Overcome Multiple Primary and Acquired Immune Resistance Mechanisms in Ovarian Cancer"

Goal: The overall objective of this study is to reprogram the ovarian tumor microenvironment with an "armed" oncolytic vaccinia virus delivering CXCR4 antagonist, PD1, and LAG3 inhibitors, while minimizing the potential for high rates of systemic toxicities.
Overlap: None

Pending Research Support

P50CA159981-07A1 (PIs: Odunsi/Moysich) 4/1/21-3/31/26
NIH/NCI
"Roswell Park Ovarian Cancer SPORE"

Goal: The overall objective of this project is to elucidate the mechanisms that contribute to tumor progression or protection in patients with chemo-resistant ovarian cancer in the face of a potent combinatorial immunotherapy approach.
Overlap: None

Active to Completed Research Support

Department of Defense (PI: Kozbor) 6/15/16-6/14/20 (no cost extension)
Investigator-Initiated Research Award W81XWH-16-1-0146
"CXCR4 Antagonist as an Adjuvant in Immunotherapy of Epithelial Ovarian Cancer"

Goal: This study examines whether soluble or tumor-targeted CXCR4 antagonists can be used as efficient adjuvants in boosting efficacies of anticancer vaccines and adoptively transferred T cells against ovarian tumors.

R03 CA223623 (PI: Kozbor) 7/16/18-6/30/20
National Cancer Institute
"The Role of CREB3L1 in Synergy between Oncolytic Vaccinia Virus and Doxorubicin"

Goal: This study examines expression of the membrane-bound transcription factor CREB3L1 in clinical specimens of ovarian tumor to explore its association with doxorubicin resistance and involvement in vaccinia virus-mediated increases in sensitivity. The insight obtained from understanding the mechanisms associated with the synergistic interaction between oncolytic virotherapy and doxorubicin may predict which patient responds to doxorubicin and if these two therapeutic agents could be combined to enhance survival and quality of life.

P30 CA016056 (PI: Johnson) 5/1/18-4/30/19
NIH Cancer Center Support Grant
National Cancer Institute
" Developmental funds for reprogramming anti-tumor immunity with oncolytic viruses"

Goal: The overall goal of this study was to prevent cancer recurrence and metastasis by eradicating CD44 antigen-expressing cancer stem cells and simultaneously target the tumor microenvironment promoting tumor growth. Using a panel of established human ovarian cancer cell lines, the aim is to investigate the plausible rationale for how CD44 increases susceptibility of cancer cells to vaccinia infection and affects induction of innate immunity.

Molecular Therapy
Oncolytics
Original Article

Recruitment of Intratumoral CD103$^+$ Dendritic Cells by a CXCR4 Antagonist-Armed Virotherapy Enhances Antitumor Immunity

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Intratumoral dendritic cells play an important role in stimulating cytotoxic T cells and driving antitumor immunity. Using a metastatic ovarian tumor model in syngeneic mice, we explored whether therapy with a CXCR4 antagonist-armed oncolytic vaccinia virus activates endogenous CD103$^+$ dendritic cell responses associated with the induction of adaptive immunity against viral and tumor antigens. The overall goal of this study was to determine whether expansion of CD103$^+$ dendritic cells by the virally delivered CXCR4 antagonist augments overall survival and *in situ* boosting with a tumor antigen peptide-based vaccine. We found that locoregional delivery of the CXCR4-A-armed virus reduced the tumor load and the immunosuppressive network in the tumor microenvironment, leading to infiltration of CD103$^+$ dendritic cells that were capable of phagocytic clearance of cellular material from virally infected cancer cells. Further expansion of tumor-resident CD103$^+$ DCs by injecting the FMS-related tyrosine kinase 3 ligand, the formative cytokine for CD103$^+$ DCs, provided a platform for a booster immunization with the Wilms tumor antigen 1 peptide-based vaccine delivered intraperitoneally with polyriboinosinic:polyribocytidylic acid as an adjuvant. The vaccine-induced antitumor responses inhibited tumor growth and increased overall survival, indicating that expansion of intratumoral CD103$^+$ dendritic cells by CXCR4-A-armed oncovirotherapy treatment can potentiate *in situ* cancer vaccine boosting.

INTRODUCTION

To be effective, cancer vaccine strategies need to promote the release of tumor antigens in the context of immunogenic tumor cell death (ICD), limit multiple levels of immunosuppression in the tumor microenvironment (TME), and increase intratumoral dendritic cell (DC) populations capable of stimulating cytotoxic T cells and driving immune responses against cancer.[1,2] Alongside traditional ICD inducers like selected chemotherapies and radiation (reviewed in Galluzzi et al.[3]), oncolytic viruses (OVs) have emerged as new members of this class of agents.[4] Oncolytic virotherapy has been recognized as a form of immunotherapy, with a herpes simplex virus expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) recently approved by the Food and Drug Administration[5] and other vectors, including vaccinia virus, undergoing extensive evaluation in multiple preclinical and clinical trials.[6–11] Although OVs have shown limited clinical efficacy as a monotherapy, emerging data suggest that combination with conventional ICD-inducing chemotherapeutic agents,[8] checkpoint inhibitors to combat PD-1/PDL-1-mediated immune suppression,[12–14] and adjuvanted vaccines[15] holds considerable promise. We have recently demonstrated that the innate resistance properties of highly metastatic ovarian tumors, together with the tumor immunosuppressive network, could be overcome by the oncolytic vaccinia virus (OVV)-delivered CXCR4 antagonist (CXCR4-A), which was particularly effective in combination with doxorubicin-mediated killing.[8] Because the CXCL12/CXCR4 axis plays multiple pleiotropic roles in the progression of ovarian cancer, including stimulation of vascular endothelial growth factor (VEGF)-mediated angiogenesis,[16] intratumoral recruitment of endothelial progenitor cells,[17] as well as accumulation of CD11b$^+$Gr1$^+$ myeloid-derived suppressor cells (MDSCs)[18] and T regulatory cells (Tregs),[19] modulation of this axis affects innate and adaptive immune mechanisms of tumor destruction by increasing T lymphocyte infiltration as well as recently reported responses to checkpoint blockers.[20] Therefore, modulation of the CXCL12/CXCR4 axis in ovarian cancer could affect multiple aspects of tumor pathogenesis, including immune dysregulation.

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Several CXCR4 antagonists have demonstrated antitumor efficacy in preclinical models and have been evaluated in early clinical trials.[21–24] However, given the abundant expression of CXCR4 by many cell types, including those of the CNS and gastrointestinal and immune systems,[25] the side effects of these antagonists need to be taken into consideration. Furthermore, the effect of soluble CXCR4 antagonists on the mobilization of CXCR4-expressing bone marrow (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.[26,27] The potential effect of delivering a CXCR4-A "payload" by OVV may also depend on the route of administration of the armed virus, affecting both intratumoral viral titers and accumulation of CXCR4-A at the tumor site or in systemic tissues. This may affect the recruitment of immune cells, including the $CD103^+$ DCs or classical type 1 DCs (cDC1s), which excel in priming and cross-presentation of tumor antigens to $CD8^+$ T cells, and $CD11b^+$ DCs or cDC2s, which are more potent at driving $CD4^+$ helper T cell responses.[28] Accumulating evidence suggests that tumor lesions enriched in type I interferon (IFN)-induced genes are also rich in T cells and that type I IFN production by the $CD103^+$ DC lineage controls spontaneous T cell priming to tumor antigens.[29] On the other hand, defective recruitment and activation of $CD103^+$ DCs leads to reduced cross-priming of $CD8^+$ T cells and poorly infiltrated or "cold" tumors.[30,31] Thus, increased myeloid cell commitment to the $CD103^+$ DC lineage and activation of intratumoral $CD103^+$ DCs could substantially enhance the effector phase of antitumor T cell responses.

Understanding the mechanisms that regulate the abundance of tumor-infiltrating lymphocytes (TILs) in the TME could unveil new therapeutic mechanisms. Because intratumoral DCs are necessary for enhanced T cell tumor responses,[2,32] we investigated the effect of the armed oncolytic virotherapy (OVV-CXCR4-A) used alone or in combination with the growth factor FMS-related tyrosine kinase 3 ligand (FLT3L; referred to hereafter as FL) on mobilization of infiltration of $CD103^+$ and $CD11b^+$ DCs to the tumor site and induction of T cell tumor responses. Using an intraperitoneal ovarian tumor model (ID8-T) enriched for $CD44^+CD117^+$ cells with a cancer stem cell-like phenotype,[6] we showed that intraperitoneal delivery of the CXCR4-A-armed vaccinia was more efficacious in inhibiting tumor growth compared with treatment with the soluble CXCR4-A (sCXCR4-A) counterpart or a systemic injection of the armed virus because of higher accumulation of the antagonist in tumors rather than in systemic tissues. The armed virotherapy treatment increased intratumoral accumulation of $CD103^+$ DCs, and their subsequent expansion by injection of the FL cytokine enhanced infiltration of antigen-experienced $CD8^+$ TILs and provided a platform for a booster immunization with the WT1 peptide-based vaccine delivered with polyriboinosinic:polyribocytidylic acid (poly(I:C)) as an adjuvant. Our studies revealed that expansion of intratumoral $CD103^+$ DCs following CXCR4 antagonist-armed oncovirotherapy treatment represents a viable approach for *in situ* therapeutic vaccination to effectively bolster antitumor immune responses.

RESULTS

Inhibition of ID8-T Ovarian Tumor Growth after Intraperitoneal or Systemic Injection of CXCR4-A Delivered as a Soluble Antagonist or by Oncolytic Virotherapy

We first assessed the effect of intravenous (i.v.) or intraperitoneal (i.p.) delivery of soluble and virally delivered CXCR4-A, expressed in-frame with the murine Fc fragment of immunoglobulin G2a (IgG2a; OVV-CXCR4-A), in C57BL/6 mice challenged i.p. with a highly metastatic syngeneic ovarian cancer cell line (ID8-T). The treatment was initiated 10 days after tumor challenge and consisted of a single injection (10^8 plaque-forming units (PFUs)/mouse) of OVV-CXCR4-A or control EGFP-expressing virus (OVV). To determine the contribution of the antagonist alone to controlling tumor growth, additional tumor-bearing mice were treated for 7 days with sCXCR4-A (10 μg/injection) delivered i.v. or i.p. or were injected with RPMI-1640 medium (control mice). Inhibition of tumor growth, quantified by bioluminescence imaging, revealed rapid tumor progression in untreated control mice (Figures S1A and S1B), with animals reaching a humane endpoint within 4 weeks of challenge (Figures 1A and 1B). Systemic delivery of OVV-CXCR4-A reduced tumor growth and extended survival compared with untreated controls ($p < 0.001$) or animals treated with the unarmed virus ($p = 0.002$; Figure S1A). On the other hand, systemic injection of sCXCR4-A demonstrated only modest effects in controlling tumor spread and extended survival by ~1 week compared with control tumor-bearing mice. The antitumor effects of the virus or soluble antagonist were more pronounced after i.p. treatment (Figure S1B). I.p. delivered OVV-CXCR4-A controlled tumor growth for 4–5 weeks, and then the tumor progressed, extending survival by over 14 days compared with mice treated with sCXCR4-A ($p < 0.001$; Figure 1B) or by ~10 days compared with the OVV-treated counterparts. A combination of the control virus and sCXCR4-A delivered either i.v. or i.p. was more efficacious in reducing tumor growth (Figures S1A and S1B) and increased survival compared with each treatment alone ($p < 0.05$; Figures 1A and 1B). The combination, however, did not achieve higher efficacy compared with a single treatment with OVV-CXCR4 (Figures 1A and 1B). This could be due to variations in the distribution of sCXCR4-A in the TME after injection compared with close contact of the antagonist with tumor stromata and cancer cells after being released from OVV-CXCR4-A-infected cancer cells. Differences in the level and physical contact of sCXCR4-A with cancer cells could directly affect tumor growth through induction of apoptosis after binding to CXCR4-expressing ID8-T cells, followed by phagocytosis of tumor cell debris by DCs (Figures S2A and S2B), a process required for induction of antitumor immune responses.[8] Thus, the more efficacious inhibition of ID-8-T tumor growth by i.p. delivery of the antagonist, either by the virus or in a soluble form, could be associated with higher concentrations of sCXCR4-A in the tumor compared with systemic delivery, as measured on day 8 after treatment ($p < 0.01$; Figures 1C and 1D). The i.p. treatment also resulted in background levels of the antagonist in sera or other organs, which was in contrast to ~2-fold higher levels of sCXCR4-A detected in sera and lymphoid organs of mice after systemic delivery. The higher concentrations of sCXCR4-A in the blood

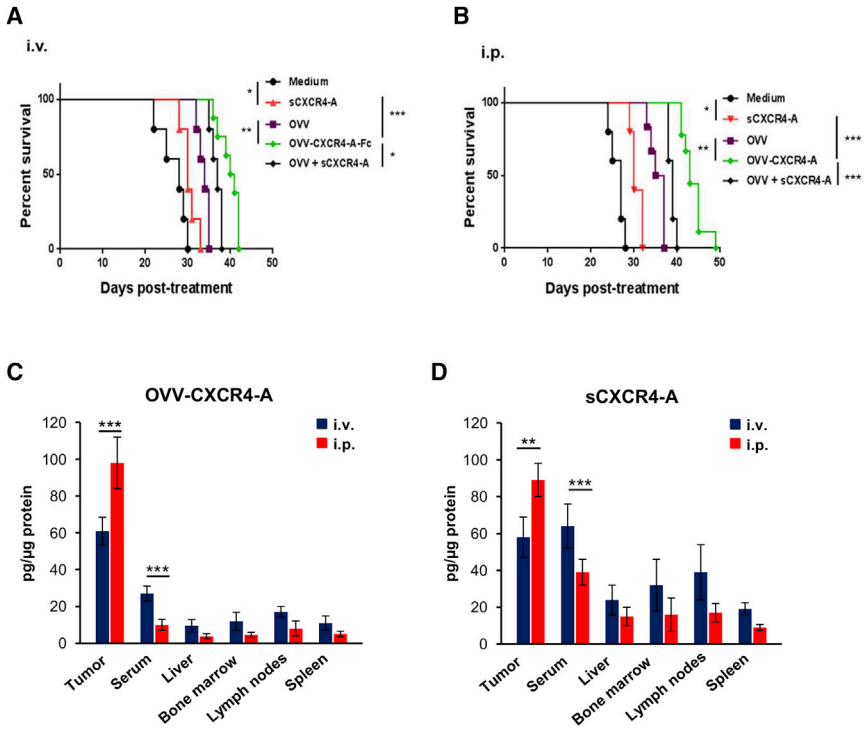

Figure 1. Inhibition of ID8-T Tumor Growth and Accumulation of sCXCR4-A in Peritoneal Washes of Tumor-Bearing Mice, Sera, and Lymphoid Organs after i.v. or i.p. Delivery of OVV-CXCR4-A and sCXCR4-A

(A and B) C57BL/6 female mice (n = 5–10 mice/group) were challenged i.p. with 3×10^5 ID8-T tumor cells and treated with sCXCR4-A (10 µg/injection for 7 days), OVV or OVV-CXCR4-A (10^8 PFU), or OVV and sCXCR4 combinations injected i.v. (A) or i.p. (B) 10 days after tumor challenge. Control mice were treated with RPMI-1640 medium. Tumor progression was monitored by bioluminescence. Kaplan-Meier survival plots were prepared, and significance was determined using the log rank method. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (C and D) Accumulation of sCXCR4-A in peritoneal washes, sera, and lymphoid organs of tumor-bearing mice after i.v. or i.p. delivery of OVV-CXCR4-A (C) or sCXCR4-A (D) to ID8-T tumor-bearing mice. Concentrations of sCXCR4-A in sera, peritoneal washes (denoted as tumors), livers, BM, lymph nodes, and spleens were determined on day 8 after treatment by ELISA after normalization to total protein content. Data are presented as the mean ± SD of five mice per group. **$p < 0.01$, ***$p < 0.001$.

and systemic tissues after i.v. injection compared with i.p. delivery were associated with ~10% increased numbers of leukocytes in the peripheral blood on days 8 and 15 before returning to baseline on day 30, although the treatment had no effect on red blood cell and platelet counts (Figures S3A–S3C).

Reduction of Intratumoral Immune Suppression and Enhanced Infiltration of CD103$^+$ DCs after OVV-CXCR4-A Treatment

Previous studies have shown that virally delivered CXCR4 antagonist blocks the CXCL12/CXCR4 axis involved in tumor progression by inhibiting local immunosuppression.[6–8,20] Therefore, we next investigated the effects of sCXCR4-A and OVV-CXCR4-A treatments on intratumoral accumulation of granulocyte-like myeloid-derived suppressor cells (G-MDSCs) and Tregs within the TME by flow cytometry analyses performed 8 days later, which roughly corresponded to the termination of viral replication *in vivo*.[6] As shown in Figure 2A, the frequencies of tumor-infiltrating CD45$^+$ leukocytes in tumor-bearing mice after virotherapy treatments were ~4-fold higher compared with those in the untreated or sCXCR4-A-treated counterparts. The antagonist, delivered i.p. as a soluble protein or secreted from virally infected tumor cells, reduced the accumulation of immunosuppressive CD11b$^+$Ly6C^{low}Ly6G^{high} G-MDSCs compared with the untreated and OVV-treated counterparts (Figure 2B; $p = 0.03$ and $p = 0.006$, respectively), and also inhibited accumulation of CD4$^+$CD25$^+$Foxp3$^+$ Tregs (Figure 2C; $p < 0.05$). Inhibition of the immunosuppressive network within the TME contributed to increased accumulation of CD8$^+$ TILs, which were detected after sCXCR4-A delivery ($p = 0.02$) and increased by over 3-fold after OVV or OVV-CXCR4-A treatment (Figure 2D; $p < 0.01$). The virotherapy-expanded CD8$^+$ TILs consisted mostly of antigen-experienced (CD44^{hi}CD62L$^+$ and CD44^{hi}CD62L$^-$) cells with less than 5% naive (CD44^{lo}CD62L$^+$) and double-negative cells (Figure 2E), which was in contrast to the predominantly naive phenotype of CD8$^+$ TILs in untreated mice. Treatment with sCXCR4-A increased the frequencies of CD44^{hi}CD62L$^+$ and CD44^{hi}CD62L$^-$ CD8$^+$ cells compared with control mice, but the changes were not significant.

The increased percentages of CD8$^+$ TILs after oncovirotherapy treatment were associated with higher infiltration of tumor-associated macrophages (TAMs) and DCs, profiled within the CD45$^+$ compartment using multi-color flow cytometry and a progressive gating strategy.[33] As shown in Figures 3A–3D, subgating all CD45$^+$ hematopoietic cells by the myeloid-specific marker CD11b that were Ly6C-negative allowed removal of neutrophils (CD11b$^+$Ly6C^{lo}) and monocytes (CD11b$^+$Ly6C^{hi}). Within the CD11b$^+$MHCII$^+$ subset, macrophages were distinguished from DCs based on CD24^{lo} and F4/80^{hi} expression, and because neither marking alone is sufficient to make this distinction,[33] these two populations were analyzed separately. Staining of the F4/80^{hi}CD24^{lo} cells with CD11b and CD11c showed that the majority of macrophages exhibited the CD11b^{hi}CD11c^{lo} phenotype, captured by the TAM1 subset of macrophages,[33] with only small proportions being double-positive for both antigens and CD11c^{hi}CD11b^{hi} in all treatment groups. The results, presented as the percentages of TAMs within CD45$^+$ cells, revealed that the relative proportions of F4/80$^+$CD11b^{hi}CD11c^{lo} cells were higher in treatment groups compared with control mice

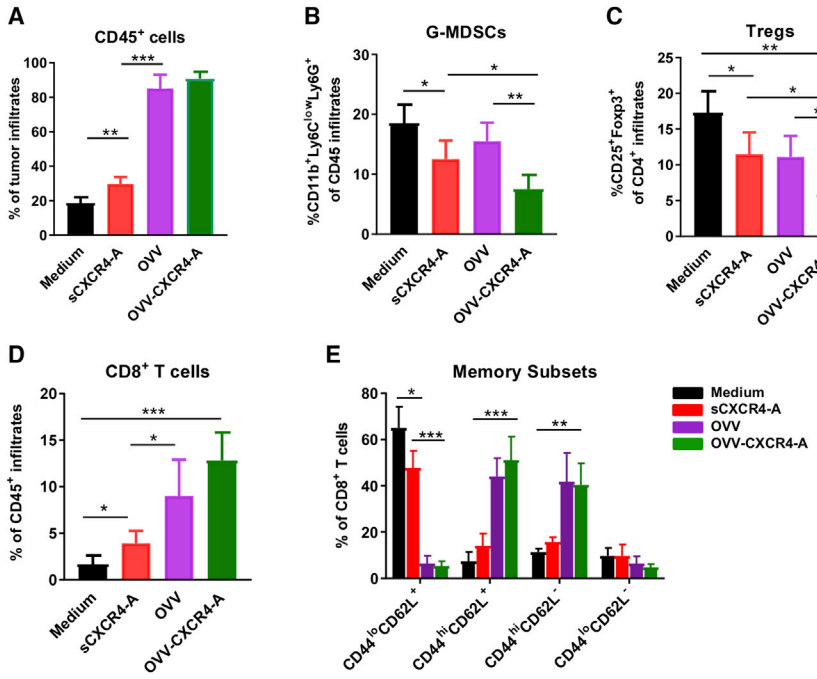

Figure 2. Increased Inflammation and Inhibition of the Immunosuppressive Network in the TME by Virally Delivered CXCR4-A

(A–D) Accumulation of leukocytes ($CD45^+$) (A), G-MDSCs ($CD11b^+Ly6G^{hi}Ly6C^{lo}$) (B), Tregs ($CD4^+CD25^+Foxp3^+$) (C), and $CD8^+$ T cells (D) in peritoneal washes of ID8-T ovarian tumor-bearing mice was analyzed by flow cytometry 8 days after treatment. (E) Memory subsets of $CD8^+$ T cells were analyzed with mAbs specific for CD44 and CD62L antigens. Background staining was assessed using isotype control antibodies. Data are mean ± SD of three or four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(Figure 3E; $p < 0.05$), whereas no significant differences were observed in the proportions of $F4/80^+CD11c^{hi}CD11b^{hi}$ cells (Figure 3F). This was in contrast to increased percentages of $CD11b^+$ and $CD103^+$ DCs within the $F4/80^{lo}CD24^{hi}$ population after virotherapy treatments (Figures 3G and 3H; $p < 0.05$) with significantly higher numbers of $CD103^+$ DCs in OVV-CXCR4-A-treated tumors compared with OVV-treated counterparts ($p = 0.04$).

FL-Mediated Expansion of Intratumoral $CD103^+$ DCs Inhibited Tumor Growth and Augmented Infiltration of $CD8^+Ly6C^+$ TILs

Given the profound association of intratumoral stimulatory DCs with patient outcome,[34] we sought to determine whether expansion of $CD103^+$ DCs in the TME would enhance the therapeutic efficacy of the combined treatment by promoting tumor antigen presentation and priming of T cells following virotherapy-mediated ICD.[35] The formative cytokine for cDC1s, which include tumoral $CD103^+$ DCs, is FL, which is predominantly produced by lymphocytes, notably natural killer cells in mouse and human tumors.[2] Because the antitumor effect of oncolytic virotherapy is short-lasting because the virus is eliminated by the innate and adaptive immune responses, we hypothesized that the paucity of $CD103^+$ DCs at the tumor site restricted the expansion of tumor-specific $CD8^+$ T cells and, therefore, limited the efficacy of the viroimmunotherapy treatment. We therefore sought to determine whether expansion of intratumoral $CD103^+$ DCs by local delivery of the FL growth factor[36] would enhance the therapeutic efficacy of the combined treatment by promoting tumor antigen presentation and priming T cells following virotherapy-mediated ICD.[35] As depicted in Figure 4A, 8 days after virotherapy treatment, tumor-bearing mice were injected i.p. with FL (5 μg/injection) for 4 days, and changes in tumor-infiltrating DCs were analyzed 2 days later by flow cytometry. As shown in Figures 4B and 4C, injection of the FL cytokine expanded over 2-fold ($p < 0.05$) the frequency of $CD103^+$ DCs among the $MHCII^+F4/80^{lo}CD24^{hi}$ cell population in both OVV and OVV-CXCR4-A-treated tumor-bearing mice (Figures 4B–4E). The combination treatment-expanded $CD103^+$ DCs were able to engulf cellular debris from OVV-exposed ID8-T cells at higher levels compared with their virotherapy-expanded counterparts (Figure 4D; $p \leq 0.04$), which is stringently required for mounting an immune response against dying tumor cells.[8] The FL-mediated increases in $CD103^+$ DCs in tumor-bearing mice were associated with decreased tumor growth (Figure 4E) compared with animals receiving monotherapy with OVV ($p = 0.04$) or OVV-CXCR4-A ($p = 0.03$).

Because intratumoral infiltration of $CD103^+$ DCs is one of the major requirements for establishing a T cell-inflamed tumor phenotype because of production of CXCL9 and CXCL10 chemokines, which promote recruitment of effector $CXCR3^+$ $CD8^+$ T cells,[37] we next examined whether this mechanism could also be used to increase survival and bolster tumor-specific T cell responses following virotherapy. For the analysis, $CD8^+$ T cells in the peritoneal cavities of control and virotherapy-treated mice were stained with antibodies specific to Ly6C antigen expressed on antigen-experienced T cells[38] as well as tetramers specific for the vaccinia virus B8R protein ($B8R\text{-}K^b$/TSYKFESV) and WT1 tumor antigen ($WT1\text{-}2D^b$/RMFPNAPYL). Figures 5A and 5B show a more than 3-fold expansion of intratumoral $CD8^+Ly6C^+$ cells after OVV treatment compared with control mice ($p < 0.001$), and the numbers increased by ~30% after FL delivery ($p < 0.05$). The increased percentages of antigen-experienced $CD8^+Ly6C^+$ TILs after OVV and FL combination treatment extended the median survival rate (45 days; Figure 5C) compared with OVV-treated and control groups of mice (33 and 27 days, respectively). Because injection of the FL cytokine into untreated mice did not affect the survival rate, it appears that virotherapy-mediated accumulation of intratumoral DCs and changes in the TME are required for the FL-mediated antitumor effect. Over 10% of $CD8^+$ TILs in mice that received oncolytic virotherapy treatments

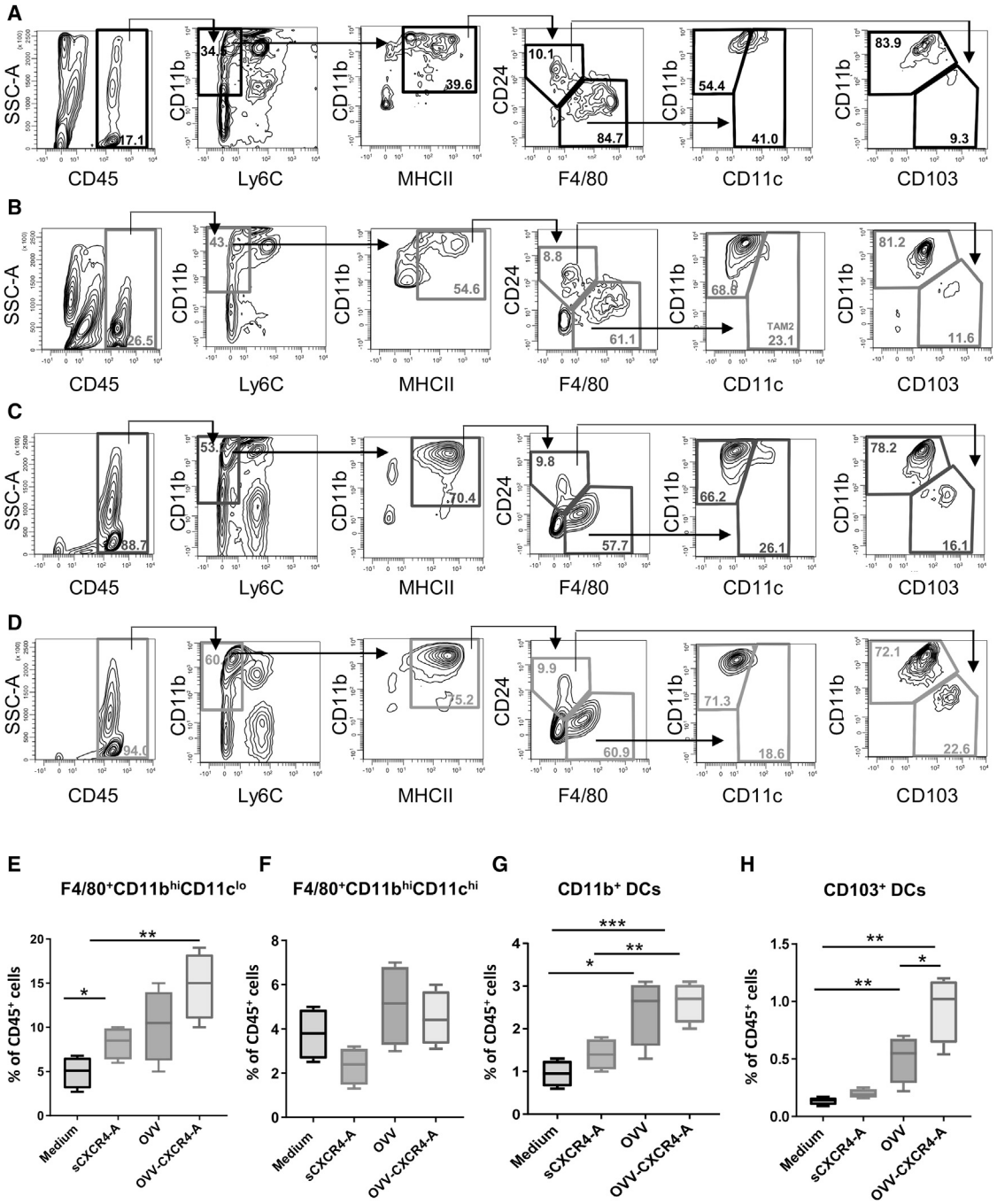

Figure 3. Intratumoral Infiltration of CD103$^+$ DCs after i.p. Treatment with Soluble or Virally Delivered CXCR4-A

(A–D) Representative flow cytometry staining and gating of myeloid cell populations infiltrating the peritoneal cavities of ID8-T-challenged mice treated with medium (A), sCXCR4-A protein (B), OVV (C), and OVV-CXCR4-A (D). (E–H) Relative proportions of tumor-infiltrating F4/80$^+$CD11b^{hi}CD11c^{lo} (E), F4/80$^+$CD11b^{hi}CD11c^{hi} (F), CD11b$^+$ DCs (G), and CD103$^+$ DCs (H) are depicted as percentages of total CD45$^+$ cells. Results are presented as mean ± SD from three or four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

were positive for the B8R-K^b/TSYKFESV vaccinia-specific tetramer, with additional increases in the percentages of tetramer-positive cells measured after FL delivery (Figures 5D and 5E). However, despite significant increases in the frequencies of CD8$^+$Ly6C$^+$ TILs, including those that were directed against the viral antigen, percentages of WT1 tetramer$^+$CD8$^+$ T cells were at background levels after

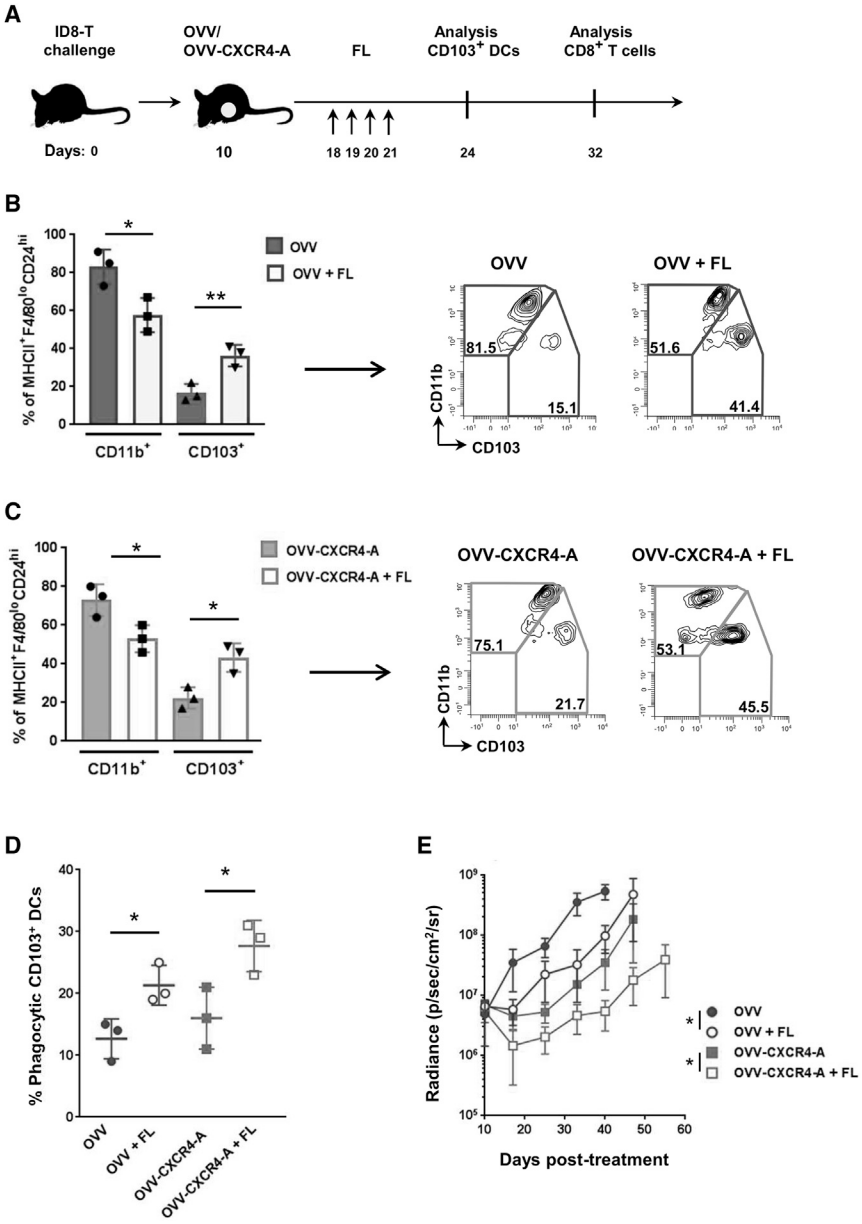

Figure 4. Expansion of Intratumoral CD103⁺ DCs by Local Delivery of the FL Cytokine Enhances the Efficacy of Oncolytic Virotherapy Treatment

(A) Graphical timeline of the treatment scheme in ID8-T tumor-bearing mice. C57BL/6 mice were injected i.p. with 3×10^5 ID8-T cells. Treatment with OVV or OVV-CXCR4-A (10^8 PFU delivered i.p.) was initiated 10 days later. To expand CD103⁺ DCs, FL was injected i.p. at 5 μg/injection for 4 consecutive days, beginning on day 8 after virotherapy treatment. Percentages of CD11b⁺ and CD103⁺ DCs in peritoneal washes of OVV- or OVV-CXCR4-A-treated, ID8-T-bearing mice (n = 3–5 mice/group) after i.p. delivered FL were analyzed 2 days later, whereas percentages of CD8⁺ TILs were assessed on day 32 by flow cytometry. (B and C) Relative proportions (left panel) and representative flow cytometry plots (right panel) of intratumoral CD11b⁺ and CD103⁺ DCs within MHCII⁺ F4/80^{lo}CD24^{hi} populations of myeloid cells infiltrating the peritoneal cavities of ID8-T tumor-bearing mice after OVV and FL treatment (B) as well as OVV-CXCR4-A and FL treatment (C). Results are presented as mean ± SD of four experiments. *p < 0.05, **p < 0.01. (D) FL-mobilized CD103⁺ DCs exhibited increased phagocytosis of tumor cell debris. CD45⁺ leukocytes isolated from peritoneal cavities of ID8-T tumor-bearing mice 2 days after treatment with OVV or OVV-CXCR4-A alone or in combination with FL were cultured with OVV-treated and CellTracker-labeled ID8-T cancer cells. After overnight incubation, the capture of tumor-associated fluorescent debris by CD103⁺ DCs was analyzed by flow cytometry. Percentages of phagocytosis of virally treated tumor cell debris by CD103⁺ DCs are presented as mean ± SD of 3 experiments. *p < 0.05. (E) Progression of ID8-T tumor growth in mice (n = 5 mice/group) treated with OVV or OVV-CXCR4-A delivered alone or in combination with the FL cytokine was monitored by bioluminescence. Data points represent mean ± SD. *p < 0.05.

oncolytic virotherapy and FL combination treatment (Figures 5F and 5G). CD8⁺Ly6C⁺ T cell responses were increased after the OVV-CXCR4-A and FL treatment combination compared with tumor-bearing mice treated with the control virus and FL ($p = 0.016$; Figures 5H and 5I) and were associated with an increased survival rate (Figure 5J). The higher percentages of B8R tetramer⁺CD8⁺ TILs in OVV-CXCR4-A-treated mice compared with those receiving the control virus with or without FL treatment ($p < 00.4$; Figure 5K) also indicated that the release of CXCR4-A from virally infected tumor cells did not interfere with migration of antigen-specific T cells to the TME, consistent with minimal expression of CXCR4-A on differentiated effector and effector memory T cells.[39] It is also notable that the percentages of B8R tetramer⁺CD8⁺ T cells in spleens and tumors, measured after single or multiple (3 times) deliveries of the oncolytic viruses, were similar (Figures S4A–S4C), possibly because of acquired resistance of residual tumors to repeated viral infections. However, despite the higher frequencies of B8R-K^b/TSYKFESV tetramer⁺CD8⁺ TILs after OVV-CXCR4-A and FL treatment compared with those generated using the control virus combination ($p < 0.05$), the percentages of WT1 tetramer⁺ CD8⁺ T cells still remained at background levels (Figure 5L). Therefore, we hypothesized that a weak expression level of the WT1 protein in ID8-T cancer cells, together with an excess of highly phagocytic macrophages in the TME, which compete for antigen availability at the tumor site, could limit the ability of CD103⁺ DCs to prime and activate sufficient numbers of WT1 tetramer⁺CD8⁺ T cells. We next investigated whether boosting the load of WT1 antigen at the tumor site with an adjuvanted WT1 peptide-based vaccine would enhance the frequencies of WT1 tetramer⁺CD8⁺ TILs.

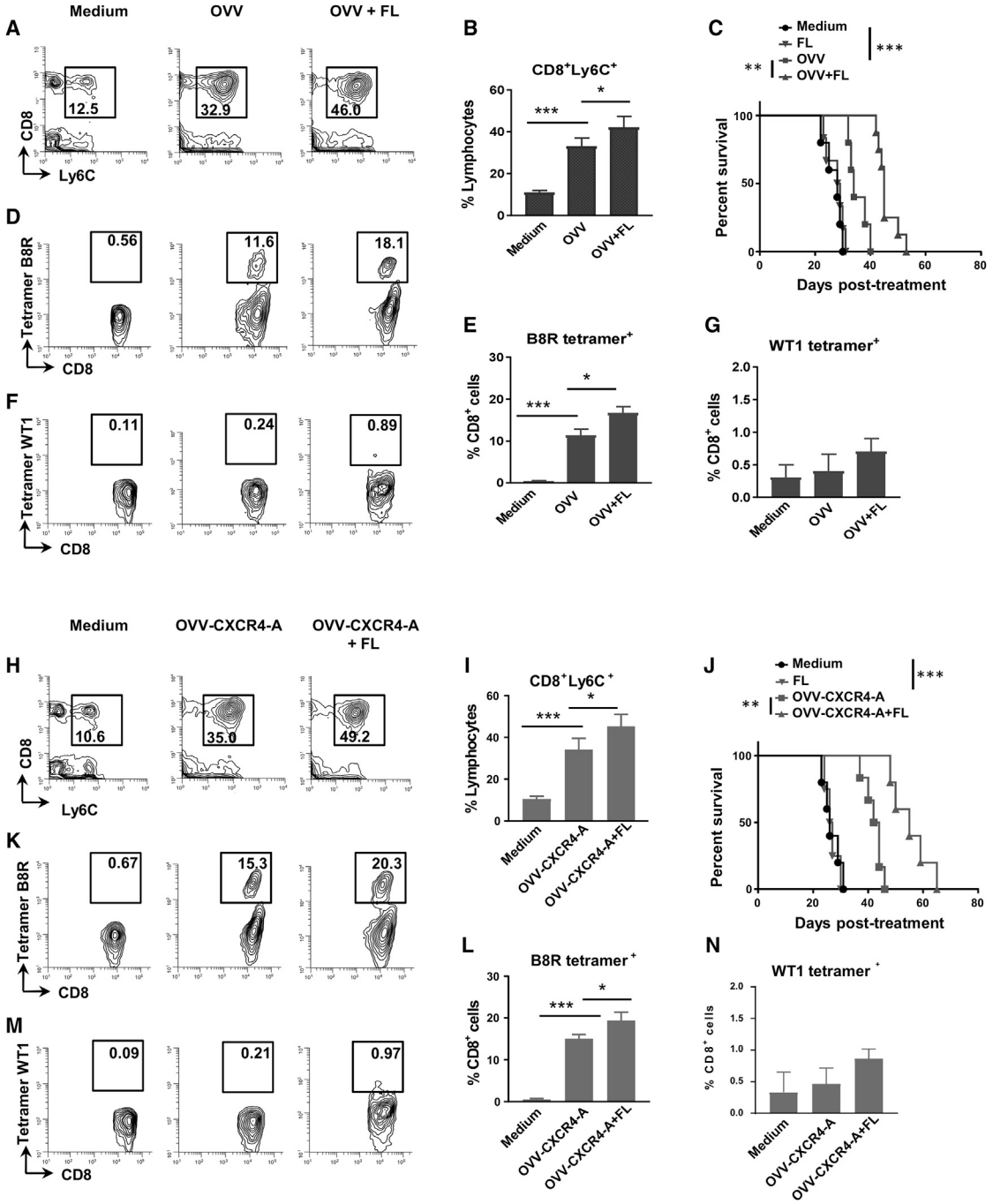

Figure 5. FL-Mediated Expansion of Intratumoral CD103$^+$ DCs Inhibits Tumor Growth and Augments Infiltration of CD8$^+$ TILs to Peritoneal Cavities of ID8-T Tumor-Bearing Mice

(A and B) Evaluation of tumor-infiltrating CD8$^+$Ly6C$^+$ T cells in peritoneal washes after OVV treatment alone or in combination with the FL cytokine (n = 5 mice/group). Representative flow cytometry staining (A) and relative proportions of CD8$^+$Ly6C$^+$ TILs (B) are shown. (C) Survival of ID8-T tumor-bearing C57BL/6 mice (n = 5–10 mice/group) after OVV and OVV plus FL treatment combinations. Survival was defined as the point where mice were killed because of extensive tumor burden. Kaplan-Meier survival plots were prepared, and significance was determined using the log rank method. $^{**}p < 0.01$, $^{***}p < 0.001$. (D and E) Representative flow cytometry staining (D) and relative proportions of a tumor-infiltrating B8R vaccinia virus-specific tetramer$^+$ subset of CD8$^+$ TILs (E). (F and G) Representative flow cytometry staining (F) and relative proportions of WT1 tetramer$^+$CD8$^+$ TILs (G). (H and I) Evaluation of tumor-infiltrating CD8$^+$Ly6C$^+$ T cells in peritoneal washes after OVV-CXCR4-A treatment alone or in combination with the FL cytokine (n = 3–5 mice/group). Representative flow cytometry staining (H) and relative proportions of the CD8$^+$Ly6C$^+$ TILs (I) regimen are shown. (J)

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Generation of WT1-Specific CD8$^+$ TILs by an Adjuvanted WT1-Peptide Vaccine Delivered after Oncovirotherapy and FL Treatment Required Batf3-Driven CD103$^+$ DCs

The WT1-specific peptide containing H2-IA^b-restricted CRYGPFGPPPSQAS and H2-D^b-restricted RMFPNAPYL epitopes[6,8] was injected i.p. to ID8-T-bearing mice (50 μg/injection) 3 days after FL delivery (Figure 6A) in combination with poly(I:C) (50 μg/injection), which binds to TLR3 expressed on CD103$^+$ DCs[40] and induces type I IFN production and DC maturation.[41,42] Additional groups of tumor-bearing mice received only virotherapy treatments before immunization to determine the importance of FL-expanded CD103$^+$ DCs in the induction of tumor-antigen-specific T cells and inhibition of tumor growth. As shown in Figure 6B, vaccination of mice after OVV and FL treatment combination exhibited potent antitumor activities, extending survival by about 15 days compared with mice treated with the virus ($p < 0.001$) and by 7–8 days compared with the virus and WT1 vaccine ($p = 0.006$). This regimen also elicited measurable WT1-specific CD8$^+$ T cell responses compared with those induced by vaccination without prior FL treatment ($p = 0.03$; Figures 6C and 6D). CXCR4-A-armed virotherapy followed by FL-mediated expansion of CD103$^+$ DCs prior to vaccination was most effective in inhibiting tumor growth (median survival of 69 days; Figure 6E) and inducing WT1-2D^b/RMFPNAPYL tetramer$^+$CD8$^+$ TILs (Figures 6F and 6G). Additional experiments performed in *Batf3*$^{-/-}$ knockout mice deficient for both CD103$^+$ and CD8α^+ DCs[29,43] revealed an absence of the WT1 vaccine-mediated protective responses (Figure 6H), stressing the need for CD103$^+$ DCs at the tumor site for induction of antitumor protective immune responses.

DISCUSSION

As cancer therapies continue to evolve and incorporate immunotherapy as an integral aspect of treatment, developing approaches that potentiate the induction of ICD and overcome non-T cell inflamed tumors will be important to realizing increased treatment efficacy. Here we showed that locoregional delivery of the CXCR4-A-armed virus is more efficacious in inhibiting orthotopic growth of ovarian tumors than i.v. injection of the unarmed counterpart, possibly because of a higher accumulation of the antagonist in the tumor than in systemic tissues. It also appears that distribution of the CXCR4 antagonist in the TME and its vicinity to both stromal and cancer cells play an important role in blocking the CXCL12/CXCR4 signaling pathway. For example, physical contact of the antagonist with the target can be more efficacious when it is released from virally infected cancer cells directly to the TME than delivered by injection because the latter form of delivery may not facilitate effective penetration in the tumor tissue. This hypothesis is consistent with higher inhibition of tumor growth by i.p. delivery of OVV-CXCR4-A virus than by injection of the soluble antagonist with a control virus by the same route. The results of our studies are in agreement with the recent work by Chen et al.,[20] demonstrating that high concentrations of localized CXCR4-A in the TME decreases immunosuppression associated with enhanced infiltration of CD8$^+$ TILs and inhibition of tumor growth. This, together with the findings that the TME may regulate clonal expansion of cancer-specific T cells[44] and that CD8$^+$ T cell proliferative responses are orchestrated by CD103$^+$ Baft3-dependent DCs[32] suggest dependence of T cell-mediated tumor regression on the intratumoral presence of CD103$^+$ DCs. Thus, therapeutic interventions that enhance infiltration of intratumoral stimulatory DCs and their capacity for driving T cell proliferation may contribute to tumor control. Among such strategies are interventions that target intratumoral TAMs and MDSCs and lead to reduced tumor burdens in preclinical models in both T cell-dependent and T cell-independent ways. For instance, inhibiting chemokine receptor type 2 (CCR2),[45] colony-stimulating factor-1 receptor (CSF-1R),[45,46] and GM-CSF[47] in preclinical models of melanoma and pancreatic, breast, and prostatic carcinoma increased intratumoral T cells and controlled tumor growth, especially when combined with anti-CTLA-4 or anti-PD-1/PD-L1. Although these studies did not determine whether the increases in T cells were a consequence of enhanced viability or proliferation, they emphasize that elements of the TME regulate the accumulation of effector T cells. In addition, the distribution of intratumoral CXCL12, which correlates inversely with that of T cells, suggests that CXCL12 is involved in T cell exclusion based on the antitumor outcome of inhibiting CXCR4. The results of our recent studies further emphasize this assumption and demonstrate that CXCR4-A-armed oncolytic virotherapy treatment was associated with increases in intratumoral accumulation of CD103$^+$ DCs and that its efficacy could be further boosted by FL-mediated expansion of CD103$^+$ DCs.

By inducing ICD and antigen release at the tumor site via viral oncolysis with simultaneous reprogramming of the TME, the armed virotherapy is personalized and can be combined with tumor-specific vaccines[48,49] after increasing the intratumoral infiltration of CD103$^+$ DCs by injection of the FL cytokine. As demonstrated here, intratumoral accumulation of CD103$^+$ DC populations at the tumor site served as a platform for the adjuvanted WT1-specific peptide vaccine booster, leading to generation of WT1 tetramer$^+$CD8$^+$ TILs and increases in overall survival. This approach could be used with a variety of tumor-associated antigens as an "off-the-shelf" product for immunization or with personalized neoantigen-specific epitopes, the presence of which has been shown to correlate with expression of immune-related genes and efficacy of checkpoint inhibitor therapy.[50] Thus, the described "*in situ* vaccination" strategy is feasible and effective in inducing and amplifying T cell responses to tumor antigens. Because a high mutational burden has been associated with an increased neoantigen load and TILs, which improved

Survival of ID8-T tumor-bearing C57BL/6 mice (n = 5–10 mice/group) after OVV-CXCR4-A and OVV-CXCR4-A plus FL treatment combination. Kaplan-Meier survival plots were prepared, and significance was determined using the log rank method. $^{**}p < 0.01$, $^{***}p < 0.001$. (K and L) Representative flow cytometry staining (K) and relative proportions of a tumor-infiltrating B8R vaccinia virus-specific tetramer$^+$ subset (L) after combined treatment with OVV-CXCR4-A and FL. (M and N) Representative flow cytometry staining (M) and relative proportions of WT1 tetramer$^+$CD8$^+$ TILs (N).

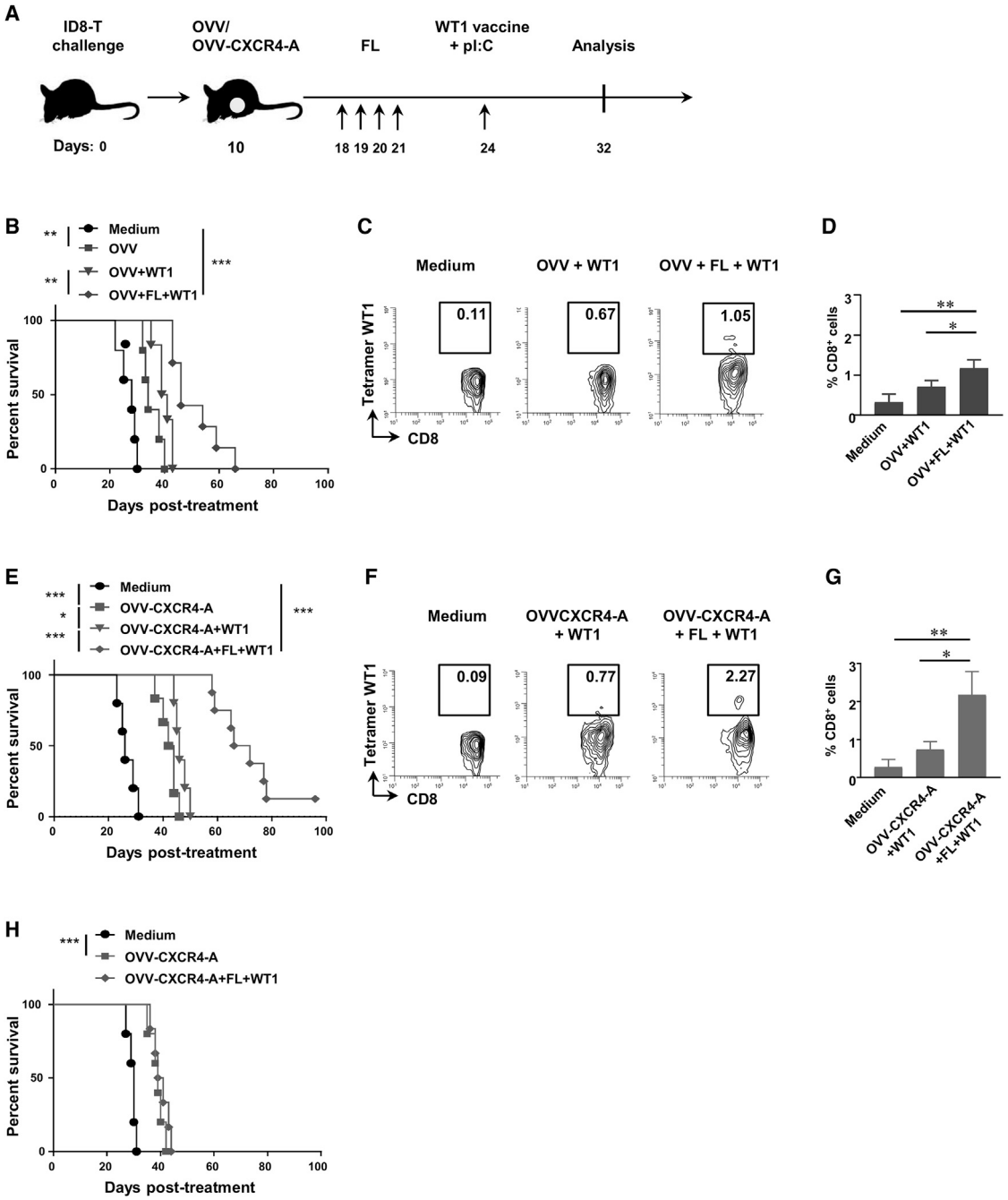

Figure 6. An Adjuvanted WT1 Vaccine Delivered after Oncovirotherapy and FL Treatment Combination Generates WT1-Specific CD8$^+$ TILs and Requires Batf3-Driven CD103$^+$ DCs

(A) Graphical timeline of the treatment scheme in ID8-T tumor-bearing mice. C57BL/6 mice were injected i.p. with 3×10^5 ID8-T cells. Treatment with OVV or OVV-CXCR4-A (10^8 PFU delivered i.p.) was initiated 10 days later. To expand CD103$^+$ DCs, FL was injected i.p. at 5 μg/injection for 4 consecutive days, beginning on day 8 after virotherapy treatment. The WT1-specific peptide was delivered i.p. (50 μg/injection) with poly(I:C) (p(I:C); 50 μg/injection) on day 3 after the last FL delivery. (B) Survival of ID8-T tumor-bearing mice (n = 5–10 mice/group) after WT1 immunization of OVV- and OVV plus FL-treated mice. Kaplan-Meier survival plots were prepared, and significance was determined using the log rank method. **$p < 0.01$. (C and D) Representative flow cytometry staining (C) and relative proportions of WT1 tetramer$^+$CD8$^+$ TILs (D) after combined treatment of OVV and WT1 vaccine as well as OVV and FL treatment followed by WT1 vaccination (n = 4–5 mice/group). (E) Survival of ID8-T tumor-bearing mice

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clinical outcomes and survival seen in patients with tumors, incorporating novel peptide sequences that result from protein-changing somatic mutation in cancer cells will be of utmost value.[51] The ability of intratumoral virotherapy to broaden the neoepitope spectrum when delivered with systemic PD-1 checkpoint inhibition, resulting in improved antitumor efficacy,[52] is consistent with the observation that oncolytic viruses may not only be used as direct tumor therapy but may also serve as a method to validate the responsiveness of T cells to predicted neoepitopes.[53]

The CXCR4-A oncolytic virotherapy-generated immunogenic tumor cell "cargo" for DC loading has the potential to be further enhanced by combination with ICD-inducing chemotherapeutic agents, such as doxorubicin, to promote improved antigen presentation to T cells[54] because of a synergistic interaction between OVV and doxorubicin.[8] This synergy could increase the amount of tumor antigens for cross-priming and broaden the diversity of danger-associated molecular patterns (DAMPs). We also found that CXCR4-A, by binding to its cognate receptor on cancer cells and inducing apoptosis, was capable of increasing phagocytosis of tumor cell debris by DCs and, therefore, appears to indirectly improve the efficacy of virotherapy. This effect could be further augmented through an interaction with the Fcγ receptors (FcγRs) on phagocytes because the antagonist, expressed as a fusion protein with the Fc portion of IgG2a, has been shown to eliminate tumor cells through the antibody-dependent cellular cytotoxicity (ADCC) mechanism,[6,7] helping to achieve the desirable induction of antitumor immunity. In such a context, high concentrations of sCXCR4-A after locoregional delivery could be relevant in immunotherapies of cancer cells with deregulated type I IFN signaling pathways[55] because FcγR-mediated antibody-dependent cellular phagocytosis bypasses the need for canonical phagocytic determinants. Such IgG-bound target cells can be efficiently processed, and the resulting tumor antigens can be used for cross-presentation by antigen-presenting cells (APCs), enhancing the repertoire of cancer antigen-directed T cell responses.[56]

Because the absence of $CD103^+$ DCs in the TME may be a critical rate-limiting step for initiating endogenous $CD8^+$ T cell responses against cancer,[31] our results argue that CXCR4-A-armed virotherapy followed by FL treatment is effective in the expansion of intratumoral $CD103^+$ DCs. The observed lack of interference of the CXCR4-A with DC infiltration is in agreement with previous studies, which showed that trafficking of DCs occurs in a coordinated, stepwise fashion, with CXCR4 and CXCL12 promoting the retention of pre-DCs in the BM but not migration to peripheral tissues and regional lymph nodes, which are directed by CCR2/CX3CR1 and CCR7, respectively.[57] Similarly, the lack or minimal expression of CXCR4 on differentiated effector T cells[58] explains the relatively high numbers of $CD8^+$ TILs expressing the Ly6C antigen, known to be associated with the effector and effector memory phenotypes.[38] Furthermore, the background levels of the CXCR4 antagonist in the blood and systemic tissues after i.p. treatment with OVV-CXCR4-A precluded any meaningful interference with the CXCR4-CCR5 interaction at the immunological synapse during T cell activation by APCs,[59] despite high CXCR4 expression on naive and central memory T cells (T_{CM}).[58] It should also be realized that CXCR4-A-armed virotherapy treatment may have a profound effect on the induction of immune cells exhaustion, in view of recent studies showing that CXCR4 inhibition improves responses to immune checkpoint blockers in mice bearing metastatic breast cancers[20] as well as decreases $CD4^+$ T cell exhaustion and improves survival in a murine model of polymicrobial sepsis.[39] Altogether, our study identifies combination therapies to potentiate ICD as well as the recruitment of $CD103^+$ DCs to tumor sites for an effective *in situ* vaccination, which holds promise for the development of more efficacious treatments for cancer patients.

MATERIALS AND METHODS

Animals and Cell Lines

Female C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA). B6.129S(C)-*Batf3*^{tm1Kmm}/J mice were purchased from The Jackson Laboratory (Sacramento, CA, USA). Experimental procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Roswell Park Comprehensive Cancer Center (RPCCC, Buffalo, NY, USA). The parental ID8 mouse ovarian epithelial cell line, derived from spontaneous malignant transformation of C57BL/6 MOSE cells,[60] and its metastatic variant ID8-T were established in our laboratory at the RPCCC.[6] Human $HuTK^-$ 143 fibroblasts, human cervical carcinoma HeLa cells, and the African green monkey cell line CV-1 were obtained from the American Type Culture Collection (Manassas, VA, USA).

Viruses

All vaccinia viruses used in this study were of the Western Reserve strain, with disrupted thymidine kinase and vaccinia growth factor genes for enhanced cancer cell specificity. The generation and characterization of OVVs expressing EGFP, the Fc portion of murine IgG2a, and CXCR4-A in the context of the Fc portion of murine IgG2a have been described.[7] The CXCR4-A fusion protein was collected in supernatants of infected $HuTK^-$ 143 cells and purified on a protein G column (GE Healthcare Life Sciences, Pittsburgh, PA, USA) as described.[7]

ELISA

Concentrations of the soluble CXCR4-A protein in sera, cell lysates from systemic tissues, and peritoneal washes of tumor-bearing mice were measured by ELISA on day 8 after treatment using plates coated with a recombinant human CXCR4 protein, MEGISIYTSDNY

(n = 5–10 mice/group) after WT1 immunization of OVV-CXCR4-A and OVV-CXCR4-A plus FL-treated mice. *$p < 0.05$, ***$p < 0.001$. (F and G) Representative flow cytometry staining (F) and relative proportions of WT1 tetramer$^+$$CD8^+$ TILs (G) after combined treatment of OVV-CXCR4-A and WT1 vaccine and OVV-CXCR4-A plus FL treatment followed by WT1 vaccination (n = 4–5 mice/group). (H) Survival of ID8-T tumor-bearing *Batf3*$^{-/-}$ female mice (n = 5) after treatment with OVV-CXCR4-A alone or in combination with FL and the WT1 adjuvanted vaccine. Kaplan-Meier survival plots were prepared, and significance was determined using the log rank method. ***$p < 0.001$.

TEEMGSGDYDSMKEPCFREENANFNKIFLPTIYS (Abcam, Cambridge, MA, USA), followed by incubation with goat anti-mouse Fc portion-specific horseradish peroxidase (HRP)-conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA), and the reaction was developed with 1-Step Ultra TMB-ELISA reagent (Thermo Fisher Scientific, Grand Island, NY, USA). In parallel, protein levels in each sample were determined by the Bradford method with protein assay dye reagent (Bio-Rad, Hercules, CA, USA).

In Vitro Phagocytosis Assays

$CD45^+$ leukocytes isolated from peritoneal cavities of ID8-T-bearing mice 2 days after virotherapy treatment alone or in combination with FL were analyzed for their ability to engulf tumor cell debris from virally treated cultures. ID8-T cells were labeled with CellTracker Blue CMF_2HC and treated with OVV at an MOI of 1 for 24 h before incubation with DCs (1:1 ratio) for 12 h. Tumor cells were treated with UV light (365 nm for 3 min) in the presence of 10 μg/mL psoralen to inactivate the virus. After overnight incubation, the capture of tumor-associated fluorescent debris by $CD103^+$ DCs was analyzed by flow cytometry. For some experiments, BM cells were flushed from the tibiae and femora of C57BL/6 mice and cultured in medium supplemented with 10 ng/mL of GM-CSF for 6 days as described.[61] After 7 days, non-adherent and loosely adherent cells were harvested, washed, and co-cultured with tumor cells labeled with CellTracker Blue CMF_2HC (Thermo Fisher Scientific, 1:1 ratio) for 12 h.

Treatments of Established Tumors

C57BL/6 mice (n = 5–10) were injected i.p. with 3×10^5 ID8-T cells. Treatments with sCXCR4-A (10 μg/injection for 7 days), OVV, and OVV-CXCR4-A (10^8 PFU), delivered i.v. or i.p., were initiated 10 days later. Tumor progression was monitored by bioluminescence imaging using the Xenogen IVIS Imaging System (PerkinElmer, Waltham, MA, USA) as described.[8] Control mice received RPMI-1640 medium or UV-inactivated virus. At the end of the experimental period, corresponding to the development of bloody ascites in control mice, tumor-bearing mice were sacrificed, and organs were examined for tumor development and metastatic spread. For *in situ* immunization of ID8-T tumor-bearing mice, the FL cytokine (BioLegend, San Diego, CA, USA) was delivered i.p. (5 μg/injection) for 4 consecutive days, beginning on day 8 after virotherapy treatment, followed by the WT1-specific peptide vaccine (amino acids [aa] 175–202; CRYGPFGPPSQASSGOARMFPNAPYL; 50 μg/injection; GenScript, Piscataway, NJ, USA) and 50 μg/mouse of poly(I:C) (Sigma-Aldrich), delivered i.p. on day 3 after the last FL injection. Progression of tumor growth was analyzed by bioluminescence.

Flow Cytometry

The induction of apoptosis or necrosis in ID8-T cells treated with sCXCR4-A (10 μg/mL), OVV, or OVV-CXCR4-A (MOI = 1) was determined by staining with Annexin V- fluorescein isothiocyanate (FITC) and LIVE/DEAD fixable violet (Thermo Fisher Scientific) according to the manufacturer's instructions. Phenotypic analysis of tumor-infiltrating myeloid cells and T cells was performed on single-cell suspensions prepared from peritoneal fluid collected 8 days after completion of the treatments. All antibodies were purchased from BD Pharmingen (San Jose, CA, USA), BD Biosciences (San Jose, CA, USA), and BioLegend, as detailed in Table S1. The phycoerythrin (PE)-labeled H-$2D^b$/RMFPNAPYL tetramer WT1 and PE-labeled H2-K^b/TSYKFESV vaccinia virus-specific tetramer B8R were obtained from the MHC Tetramer Production Facility (Baylor College of Medicine, Houston, TX, USA). Percentages of $CD4^+$ T cells expressing Foxp3 were determined by intracellular staining using the BD Pharmingen Transcription Factor Buffer Set (BD Biosciences) according to the manufacturer's protocol. For tetramer analysis, lymphocytes were also gated on cells that were negative for CD11b expression. Background staining was assessed using isotype control antibodies. Before specific antibody staining, cells were incubated with Fc blocker (anti-CD16/CD32 mAb) for 10 min and analyzed on the LRS II flow cytometer (BD Biosciences). Data analysis was performed using WinList 3D 7.1 (Verity Software House, Topsham, ME, USA).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad, La Jolla, CA, USA). Unless otherwise noted, data are presented as mean ± S.D. combined with unpaired, two-tailed Student's t test. Kaplan-Meier survival plots were prepared, and median survival times were determined for tumor-challenged groups of mice. Statistical differences in survival across groups were assessed using the log rank Mantel-Cox method. The threshold for statistical significance was set to $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2019.06.003.

AUTHOR CONTRIBUTIONS

A.M., M.P.K., M.G., and M.A.G. performed the experiments. M.O., K.O.O., A.J., and H.R. designed the experiments and performed data analysis and manuscript review. D.K. designed the experiments and analyzed and interpreted the data. M.P.K. and D.K. drafted the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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OMTO, Volume *14*

Supplemental Information

Recruitment of Intratumoral CD103^{+} Dendritic Cells by a CXCR4 Antagonist-Armed Virotherapy Enhances Antitumor Immunity

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SUPPLEMENTAL FIGURES

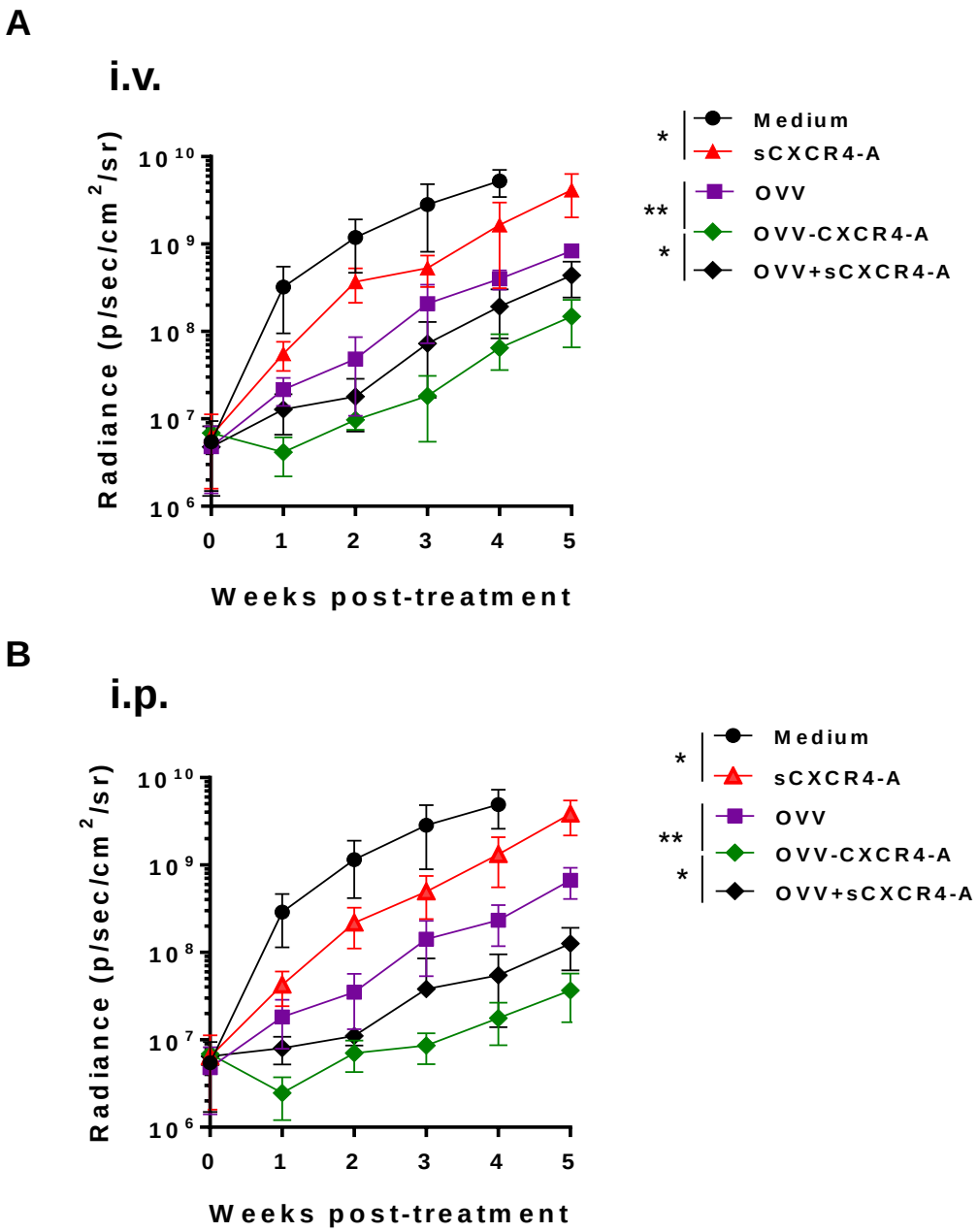

Figure S1. Local delivery of CXCR4-A is more effective at inhibiting tumor growth than systemic treatment. (**A, B**) C57BL/6 female mice (*n* = 6 - 10 per group) were challenged i.p. with $3x10^5$ ID8-T tumor cells. The tumor-bearing mice were treated with sCXCR4-A protein (10 mg/injection for 7 days), OVV, or OVV-CXCR4-A (10^8 PFU) injected i.v. (**A**) or i.p. (**B**) 10 days after tumor challenge. Control mice were treated with RPMI-1640 medium. Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. **P* < 0.05, ***P* < 0.01.

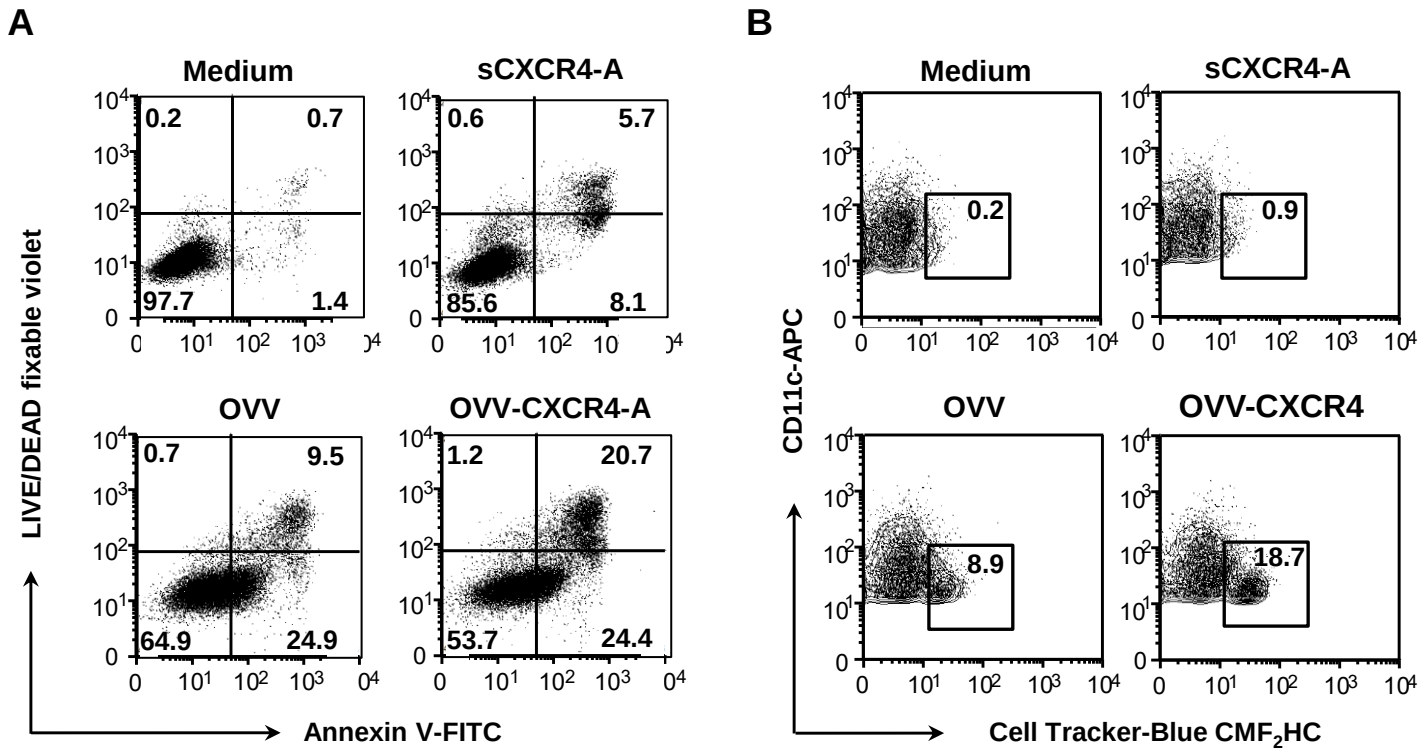

Figure S2. The CXCR4-A armed-virotherapy induces apoptosis associated with phagocytosis of tumor cell debris by DCs and delays tumor growth after FL-mediated expansion of CD103$^+$ DCs. (**A**) Cell death of ID8-T tumor cells treated with medium, sCXCR4-A (10 mg/ml), OVV or OVV-CXCR4-A (MOI = 1) was determined by staining with Annexin V-FITC and LIVE/DEAD fixable violet to measure the induction of early apoptosis (Annexin V$^+$/LIVE/DEAD fixable violet$^-$) and late apoptosis/necrosis (Annexin V$^{+/-}$/LIVE/DEAD fixable violet$^+$) by flow cytometry 24 h later. One representative experiment out of three performed is shown. (**B**) Phagocytosis of cell-tracker-blue CMF_2HC-labeled ID8-T tumor cells treated with medium, sCXCR4-A, OVV, or OVV-CXCR4-A by BM-derived, CD11c$^+$ DCs. Tumor cells were labeled with tracker-blue CMF_2HC before treatment and, after inactivating the virus, co-cultured with BM-derived DCs (1:1 ratio) for 12 h followed by staining with CD11c-APC antibody and flow cytometry analysis of double-positive cells. The percentages of CD11c-expressing DCs taking up tumor cell debris are indicated. One representative experiment of three independent experiments performed is shown.

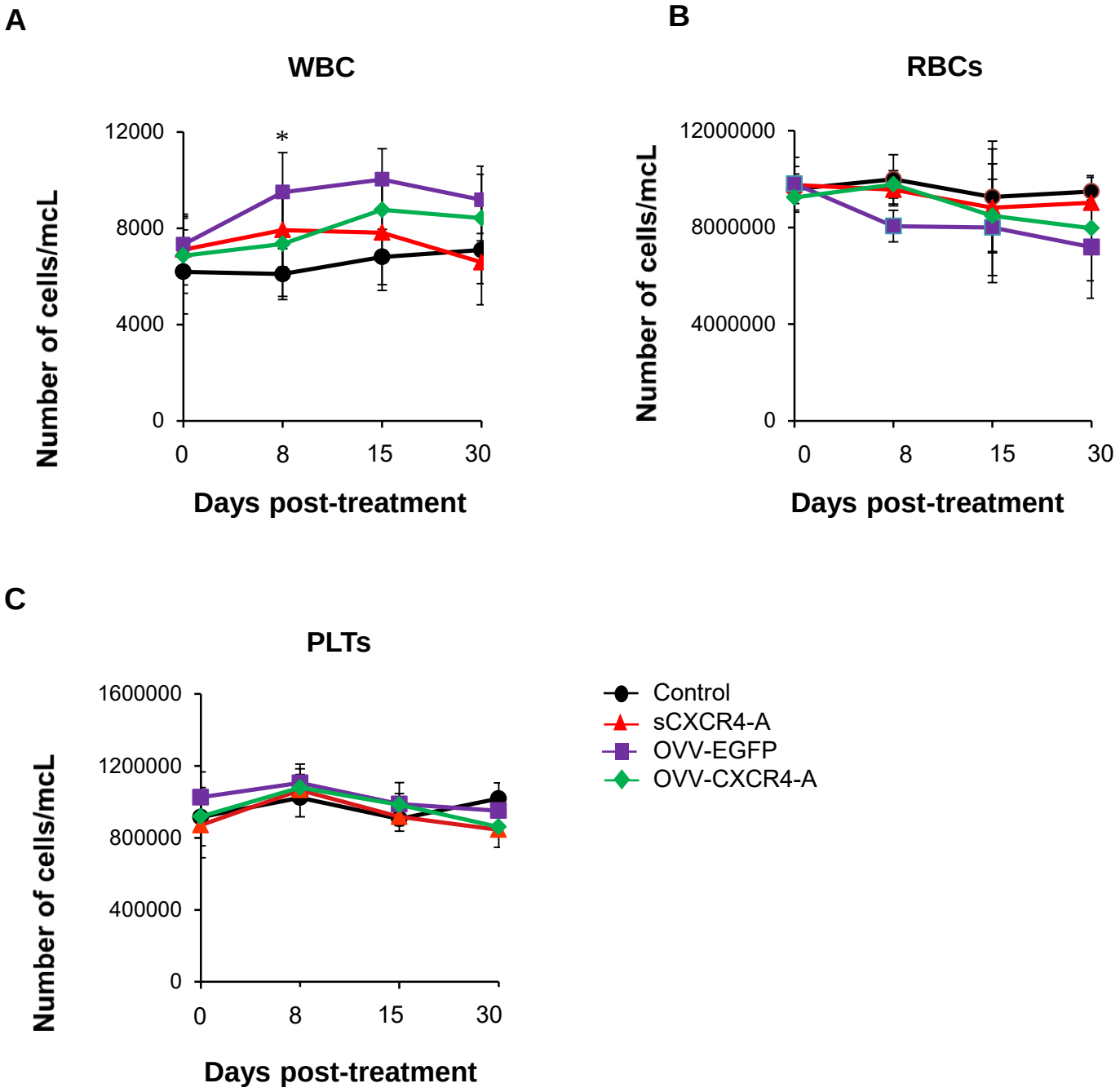

Figure S3. Cell blood count (CBC) after treatment with CXCR4 antagonist-armed OVV or sCXCR4-A delivered i.v. to ID8-T tumor-bearing mice. Mice (n = 5) were bled from the retro-orbital sinus to obtain complete counts of WBCs (**A**), RBCs (**B**), and PLTs (**C**) before treatment and on days 8, 15 and 30 after treatment initiation. The numbers of WBCs, RBCs, and PLTs in the heparinized blood samples were determined using IDEXX ProCyte Dx Hematology analyzer (IDEXX Laboratories, Inc., Westbrook, ME). Data are presented as the mean ± SD of five mice per group. *P < 0.05.

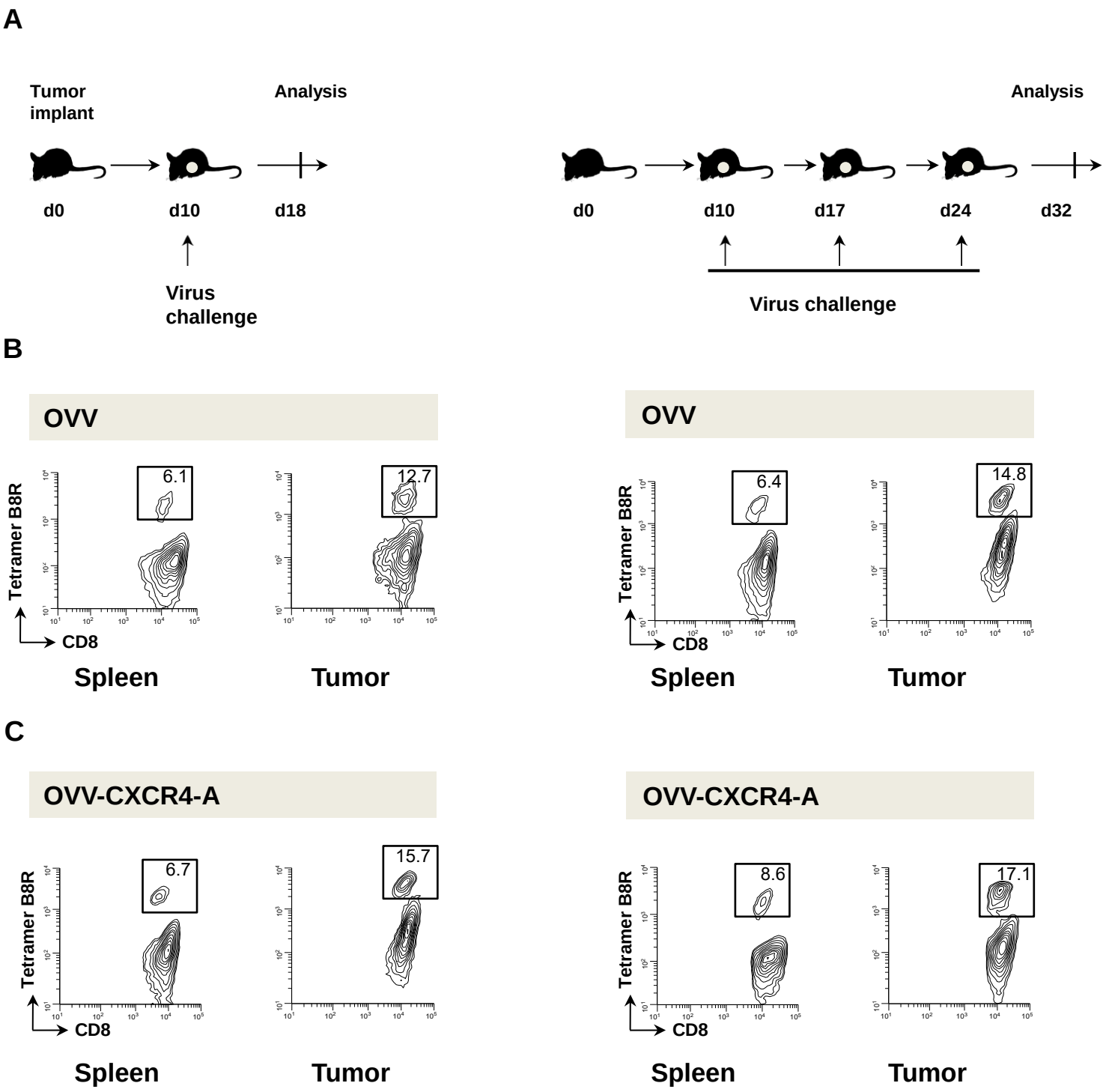

Figure S4. The effect of a single or multiple oncolytic virotherapy treatment on accumulation of B8R tetramer$^+$CD8$^+$ T cells in spleen and peritoneal cavities of ID8-T tumor-bearing syngeneic mice. (**A**) Graphical time line of the treatment scheme in ID8-T tumor-bearing mice. C57BL/6 mice were injected i.p. with 3 x 10^5 ID8-T cells. Treatment with OVV or OVV-CXCR4-A (10^8 PFU delivered i.p.) was initiated 10 days later. An additional group of mice was injected three times with the oncolytic viruses in a weekly intervals. The accumulation of B8R tetramer$^+$CD8$^+$ T cells in spleen and peritoneal cavities after treatment with OVV (**B**) or OVV-CXCR4-A (**C**) was analyzed by flow cytometry 8 days after treatment completion. One representative experiment of three independent experiments performed is shown.

SUPPLEMENTAL TABLE

Antibody	Clone	Source
CD45-V450	30-F11	BD Pharmingen
CD45-PerCP-Cy5.5	30-F11	BD Pharmingen
CD4-PE	GK1.5	BD Pharmingen
CD8a-BV786	53-6.7	BD Pharmingen
CD11b-BV786	M1/70	BD Pharmingen
Ly6G-PE	1A8	BD Pharmingen
Ly6C-FITC	AL-21	BD Pharmingen
CD11c-APC	HL3	BD Pharmingen
CD103-BV421	M290	BD Pharmingen
CD24-Alexa Fluor 700	M1/69	BD Pharmingen
CD25-FITC	PC61	BioLegend
I-A/I-E-BV605	M5/114.15.2	Biolegend
F4/80-PE	BM8	BioLegend
Foxp3-Alexa Fluor 647	MF-14	BioLegend
CD45R/B220	RA3-6B2	BD Biosciences
CD44-PerCP-Cy5.5	IM7	BD Pharmingen
CD62L-PE-Cy7	MEL-14	BD Pharmingen

Table S1. Monoclonal antibodies used in flow cytometry analysis.

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