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14. ABSTRACT Oncolytic viruses are capable of *in situ* vaccination as they induce immunogenic cell death in cancer cells and release tumor associated antigens for priming of tumor-specific $CD8^+$ T cells by tumor-infiltrating $CD103^+$ dendritic cells (DCs). Using a metastatic ID8-T ovarian tumor model in syngeneic mice, we explored whether expansion of $CD103^+$ DCs following a CXCR4 antagonist-armed oncolytic vaccinia virus augments *in situ* booster immunization with a cancer peptide-based vaccine. We found that intratumoral delivery of the armed virus reduced tumor load and the immunosuppressive network leading to increased infiltration of $CD8^+$ T cells and phagocytic $CD103^+$ DCs at the tumor site. Expansion of the tumor-residing $CD103^+$ DC population by injection of the growth factor FLT3L into peritoneal cavities of ID8-T tumor bearing mice provided a platform for subsequent boost with a peptide-based adjuvanted vaccine that elicited potent $CD8^+$ T cell responses and inhibited tumor growth. Our results revealed that expansion of intratumoral $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy treatment enhanced *in situ* booster immunization with an adjuvanted tumor-specific peptide-based vaccine and improved therapeutic efficacy.

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1. INTRODUCTION:

Effective *in situ* vaccination uses local or intratumoral immunomodulation to induce immunogenic tumor cell death (ICD) to release tumor-associated antigens (TAAs) from tumor cells, inhibit multiple levels of immunosuppression in the tumor microenvironment (TME), and increase infiltration of intratumoral antigen presenting cells (1). Oncolytic viruses are capable of *in situ* vaccination as they induce ICD in cancer cells and release TAAs for priming of tumor-specific $CD8^+$ T cells by tumor-infiltrating $CD103^+$ dendritic cells (DCs). Using a metastatic ID8-T ovarian tumor model in syngeneic mice, we explored whether expansion of $CD103^+$ DCs following a CXCR4 antagonist-armed oncolytic vaccinia virus treatment could augment *in situ* booster immunization with a cancer peptide-based vaccine. We found that intratumoral delivery of the armed virus reduced tumor load and the immunosuppressive network leading to increased infiltration of $CD8^+$ T cells and $CD103^+$ DCs at the tumor site. The latter cells were capable of phagocytic clearance of cellular debris from virally-infected ID8-T tumor. Expansion of the tumor-residing $CD103^+$ DC population by injecting the growth factor FLT3L provided a platform for boost with a peptide-based adjuvanted vaccine. Intraperitoneal vaccination with the Wilms' tumor antigen 1 (WT1)-specific T cell helper and cytotoxic T cell (CTL) epitopes delivered with Toll-like receptor 3 (TLR3)-specific agonist, polyriboinosinic:polyribocytidylic acid (polyI:C), increased the antitumor efficacy. The treatment-mediated inhibition of tumor growth was associated with increased intratumoral recruitment of WT1 tetramer$^+$$CD8^+$ T cells, which were harbored within the antigen-experienced $Ly6C^+$ T cell population. Our results revealed that expansion of intratumoral $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy treatment enhances *in situ* booster immunization with an adjuvanted tumor-specific epitope-based vaccine and elicits potent and sustained anti-tumor immunity at the tumor site, thus achieving better therapeutic efficacy.

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

2. KEY WORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Body (from Original SOW): Specific Aim 1. To design an effective DC vaccine against ID8-T ovarian tumor in syngeneic mice, we have analyzed whether expansion of intratumoral $CD103^+$ DCs after treatment with a CXCR4 antagonist-armed oncolytic virotherapy augments *in situ* peptide-based vaccination.

Task 1. We have compared antitumor efficacy of soluble and virally-delivered CXCR4 antagonist against a highly metastatic variant of the murine epithelial ovarian cancer cell line ID8-T growing orthotopically in syngeneic C57BL/6 mice. The CXCR4 antagonist, expressed in the context of the murine Fc fragment of IgG2a by oncolytic vaccinia virus (OVV-CXCR4-A-Fc) or as a soluble protein (CXCR4-A-Fc) was delivered intravenously (i.v.) or intraperitoneally (i.p.) to syngeneic mice after orthotopic challenge with metastatic ID8-T ovarian tumor cells.

Progress:

1 a. Accumulation of the CXCR4-A-Fc Antagonist in Tumor Tissues after Systemic or Intratumoral Delivery and Its Effect on Inhibition of Tumor Growth.

The oncovirotherapy treatment was initiated 10 days after tumor challenge and consisted of a single injection (10^8 PFU/mouse) of OVV-CXCR4-A-Fc or control virus expressing the enhanced green fluorescence protein (OVV-EGFP). To determine the contribution of the antagonist alone to tumor regression, additional groups of tumor-bearing mice were treated for 7 days with the sCXCR4-A-Fc fusion protein (10 μg/injection) or were injected with PBS (control group). Progression of tumor growth, quantified by bioluminescence imaging,

revealed rapid tumor cell dissemination in untreated, control mice (**Fig. 1*A***) that were killed within 4 weeks after tumor challenge (*SI Appendix* Fig. S1*A,B*). Systemic delivery of OVV-CXCR4-A-Fc reduced tumor progression and significantly extended survival compared with control mice ($P < 0.0001$) or animals treated with OVV-EGFP ($P = 0.002$; Fig. S1*A*). On the other hand, the systemic delivery of sCXCR4-A-Fc was not very effective in controlling tumor spread and extended survival by ~1 week compared with control mice. The study revealed that antitumor effects of the virotherapy or soluble antagonist were more pronounced after intratumoral delivery (**Fig. 1*B***). Among the treatment approaches, the best efficacy was achieved with the armed virus delivered i.p. The OVV-CXCR4-A-Fc treatment resulted in ~4-wk tumor regression after which period tumor growth continued at a rate similar to that in control mice, extending the survival by ~2 weeks compared to mice treated with sCXCR4-A-Fc ($P < 0.001$; Fig. S1*B*) or one week compared to the OVV-EGFP-treated counterparts. Histology performed on formalin-fixed omental tumor tissue samples obtained thirty days after each treatment revealed infiltration of leukocytes after OVV-EGFP or OVV-CXCR4-A-Fc treatment. This contrasted with the poorly inflamed tumors in control or sCXCR4-A-Fc-treated mice (*SI Appendix*, Fig. S2*A,B*), indicating that the increased inflammation at the tumor site was largely induced by the viral treatment. The intratumoral delivery of the antagonist either by the virus or in a soluble form resulted in higher concentrations of the sCXCR4-A-Fc fusion protein in the tumor compared with those measured after the systemic delivery ($P < 0.001$), and almost background levels of the antagonist in sera or other organs (**Fig. 1*C,D***). On the other hand, similar levels of the sCXCR4-A-Fc antagonist were detected in tumor tissues, sera and lymphoid organs after the systemic delivery. The higher concentrations of the antagonist in systemic tissues contributed to increased numbers of leukocytes in the peripheral blood by ~10% on days 8 and 15 before returning to the baseline on day 30, and had no effect on red blood cell and platelet counts (*SI Appendix*, Fig. S3A-*C*).

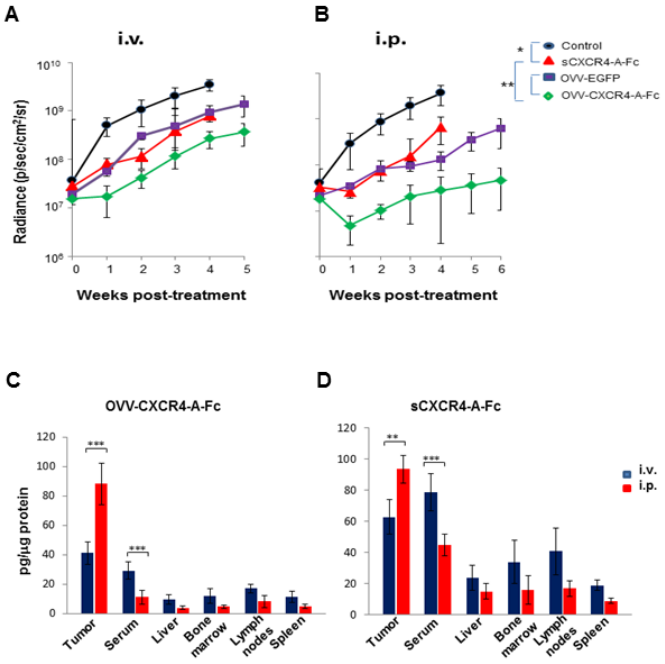

Figure 1. Fig. 1. Antitumor activity of the CXCR4 antagonist-armed oncolytic virotherapy against orthotopic ID8-T ovarian tumor in syngeneic mice. C57BL/6 female mice ($n = 8$) were challenged i.p. with ID8-T tumor cells ($3x10^5$ cells). The tumor-bearing mice were treated with sCXCR4-A-Fc (10 µg/injection for 7 days), OVV-EGFP, or OVV-CXCR4-A-Fc (10^8 PFU) injected i.v. (***A***) and i.p. (***B***) 10 days after tumor challenge. Control mice were treated with PBS. Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. *$P < 0.05$, **$P < 0.01$. Accumulation of the CXCR4-A-Fc antagonist in tumor tissues, sera and lymphoid organs after i.v. (***C***) or i.p. (***D***) delivery of OVV-CXCR4-A-Fc or the s CXCR4-A-Fc fusion protein to ID8-T tumor-bearing mice. Concentrations of CXCR4-A-Fc fusion protein were determined on day 8 after the treatment by ELISA in sera, and cell lysates of tumor, liver, BM, lymph nodes, and spleen tissues after normalization to total protein content. Data are presented as the mean ± SD of five mice per group. **$P < 0.01$, ***$P < 0.001$.

1 b. CXCR4 Antagonist Inhibits the Immunosuppressive Network and Enhances Intratumoral Infiltration of $CD8^+$ T cells and $CD11b^+$ Myeloid Cells.

The reduced antitumor efficacy of the control virus compared to that mediated by the CXCR4 antagonist-armed vaccinia after i.p. delivery, despite similar numbers of tumor-infiltrating $CD45^+$ leukocytes (**Fig. 2*A***), emphasized the importance of blocking the CXCL12/CXCR4 signaling axis to inhibit the immunosuppressive network in the TME. We next investigated the effect of the CXCR4 antagonist delivered as a soluble protein or secreted from virally-infected tumor cells on intratumoral accumulation of granulocytic myeloid-derived suppressor cells (G-MDSCs), plasmacytoid DCs (pDCs), and T regulatory cells (Tregs). Flow cytometry analysis performed on day 8 after completion of the treatment reveal that i.p. delivery of the sCXCR4-A-Fc antagonist resulted in significant reductions of intratumoral infiltration of G-MDSCs ($CD11b^+Ly6C^{low}Ly6G^{high}$) and pDCs ($B220^{high}Ly6C^{high}CD11c^{low}$) compared to control mice (**Fig. 2*B,C***; $P = 0.003$ and 0.05, respectively). A similar profile of treatment-induced responses was measured for the tumor-infiltrating $CD4^+CD25^+Foxp3^+$

Tregs although the changes did not reach a significant level (**Fig. 2*D***). Delivering the antagonist to the tumor-bearing mice by vaccinia virus further decreased accumulation of the immunosuppressive elements compared to the treatment with the soluble antagonist or control virus (**Fig. 2*D***). These changes contributed to significantly higher accumulation of both $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes (TILs) in virally-treated mice compared with control animals (**Fig. 2*E,F***; $P = 0.01$ and $P = 0.004$, respectively), predominantly with the effector memory phenotype ($CD44^{hi}CD62L^-$; *SI Appendix*, Fig. S4*A*, *B*). The increased percentages of TILs after oncovirotherapy treatment was also associated with higher infiltration of tumor-associated macrophages (TAMs) and DCs. To characterize their diversity, we profiled the tumor-infiltrating $CD45^+$ compartment using multi-color flow cytometry and progressive gating strategy (1). Subgating all $CD45^+$ hematopoietic cells by the myeloid-specific marker CD11b that were Ly6C-negative allowed removal of neutrophils ($CD11b^+Ly6C^{lo}$) and $CD11b^+$ $Ly6C^{high}$ monocytes. Within the $CD11b^+MHCII^+$ population, TAMs were distinguished from DCs based on high F4/80 expression and low CD24 levels. The flow cytometry analysis revealed that the control and CXCR4 antagonist-armed viruses significantly increased intraperitoneal recruitment of $CD11b^{hi}CD11c^{lo}$ TAM1 and $CD11b^{lo}CD11c^{hi}$ TAM2 compared to the untreated or sCXCR4-A-Fc-treated mice (**Fig. 2*G,H***; $P < 0.03$). Parsing of the $F4/80^{lo}CD24^{hi}$ compartment revealed two populations of DCs based on differential expression of CD11b and CD103, similar to observations made in healthy peripheral tissues (2). As shown in **Fig. 2*I,J***, expression of the CXCR4 antagonist in the TME of virally-treated tumors had no effect on infiltration of $CD11b^+$ DCs but increased accumulation of $CD103^+$ DCs compared to OVV-EGFP-treated counterparts ($P = 0.04$), whereas the soluble CXCR4 antagonist had no significant effect on intratumoral accumulation of either TAMs or DCs compared to control tumors.

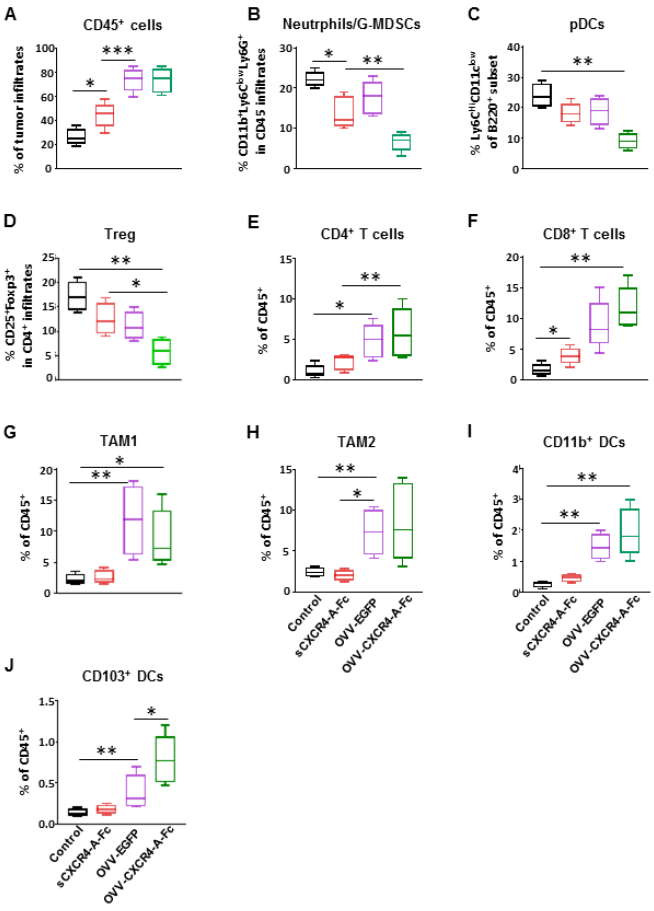

Fig. 2. Evaluation of immune infiltrates in ascites-derived tumors or peritoneal washes after treatments with the CXCR4 antagonist delivered as the sCXCR4-A-Fc fusion protein or by OVV-CXCR4-A-Fc oncolytic virotherapy. Additional tumor-bearing mice were treated with OVV-EGFP or PBS. Recruitment of leucocytes ($CD45^+$) (***A***), neutrophils/G-MDSCs ($CD11b^+Ly6G^{hi}Ly6C^{lo}$) (***B***), pDCs ($B220^{hi}Ly6C^{hi}$ $CD11c^{lo}$) (***C***), Tregs ($CD4^+CD25^+Foxp3^+$) (***D***), $CD4^+$ (***E***) and $CD8^+$ (***F***) T cells, TAM1 ($CD45^+CD11b^{hi}$ $Ly6C^-MHCII^+F4/80^+CD11c^{lo}$) (***G***), TAM2 ($CD45^+$ $CD11b^{lo}Ly6C^-MHCII^{hi}F4/80^{hi}CD11c^{hi}$) (***H***), $CD11b^+$ DCs ($CD45^+CD11b^{hi}Ly6C^-MHCII^{hi}F4/80^{lo}CD24^{hi}$ $CD103^{lo}$) (***I***), $CD103^+$DCs ($CD45^+CD11b^{lo}LY6C^-$ $MHCII^+F4/80^{lo}CD24^{hi}CD103^{hi}$) (***J***) were analyzed by flow cytometry 8 days after treatments, as described in the Materials and Methods. Data are mean ± SD of three or four independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

Task 2. We have analyzed the ability of soluble and virally-delivered CXCR4 antagonists to induce immunogenic cell death (ICD) in ID8-T tumor cells and phagocytosis of tumor cell debris by DCs.

In view of the previously demonstrated induction of ICD by vaccinia virus in cancer cells (3-5) and ability of $CD103^+$ DCs to transport intact TAAs to the tumor-draining lymph node (6), the increased intratumoral infiltration of $CD103^+$ DCs together with lower accumulation of the immunosuppressive elements in OVV-

CXCR4-A-Fc-treated tumors compared to those receiving the control virus could contribute to the improved immunogenic potential of the armed virotherapy (7). In addition, the soluble CXCR4 antagonist secreted from virally-infected cells could augment vaccinia-mediated killing of uninfected tumor cells by binding to CXCR4-expressing ID8-T cells and inducing apoptosis. To address this hypothesis, we investigated the CXCR4 antagonist-induced apoptosis and necrosis in ID8-T cells followed by phagocytosis of tumor cell debris by bone marrow (BM)-derived DCs as well as tumor-derived $CD103^+$ DCs.

Progress:

2 a. Expansion of $CD103^+$ DCs at the Tumor Site by Growth Factor FLT3L Increased Phagocytosis of Tumor Cell Debris by $CD103^+$ DCs.

We first analyzed the induction of apoptosis and necrosis in 24-h cultures of ID8-T cancer cells treated with 10 μg/ml of sCXCR4-A-Fc, a concentration of the antagonist that accumulates in 24-h supernatants of OVV-CXCR4-A-Fc-infected cultures. Additional conditions included tumor cells infected with OVV-Fc or OVV-CXCR4-A-Fc at multiplicity of infection (MOI) of 1. The induction of apoptosis/necrosis analyzed by flow cytometry with Annexin V-FITC and LIVE/DEAD fixable violet revealed that while sCXCR4-A-Fc antagonist induced apoptosis in ~15% of cells, the OVV-CXCR4-A-Fc treatment increased the number of apoptotic cells to ~45%, which was significantly higher compared to those in cultures treated with the control virus (**Fig. 3*A***; $P < 0.001$). The treatment-induced changes in apoptotic/necrotic cells also affected the engulfment of tumor cell debris by DCs. As shown in **Fig. 3*B***, phagocytosis of OVV-CXCR4-A-Fc-treated ID8-T cells by $CD11c^+$ BM-derived DCs was more effective compared to the soluble antagonist- and OVV-Fc-treated culture ($P < 0.0001$ and $P = 0.02$, respectively), suggesting that the CXCR4-A-Fc fusion protein released for the virally-infected tumor cells directly augmented the antitumor immune responses induced by the armed vaccinia virus in the tumor-bearing mice because immunogenic phagocytosis is stringently required for mounting immune response against dying tumor cells *in vivo* (7-11).

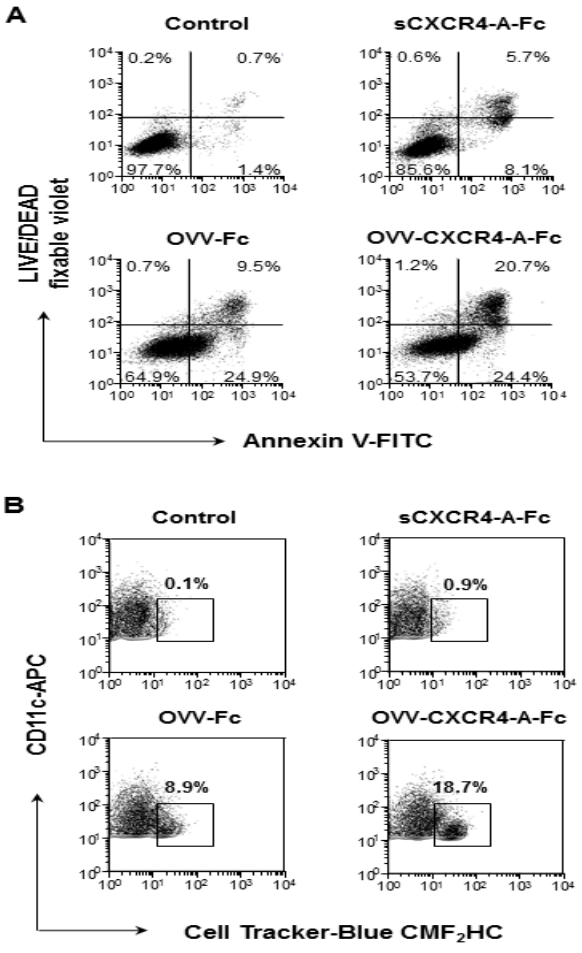

Fig. 3. The CXCR4 antagonist induces apoptosis associated with phagocytosis of tumor cell debris by BM-derived $CD11c^+$ DCs. (***A***) Cell death of ID8-R tumor cells treated with the sCXCR4-A-Fc fusion protein (10 μg/ml), OVV-Fc or OVV-CXCR4-A-Fc (MOI = 1) was determined by staining with Annexin V-FITC 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 CMF2HC-labeled ID8-T tumor cells treated with the sCXCR4-A-Fc fusion protein, OVV-Fc, or OVV-CXCR4-A-Fc by $CD11c^+$ DCs was measured after 12 h of incubation by flow cytometry. All tumor cell cultures were treated with UV and psolaren to eliminate the virus before combining with DCs. The percentages of CD11c-expressing DCs taking up tumor cells are indicated. One representative experiment of three independent experiments performed is shown.

Results of the *in vitro* analysis of phagocytosis of virally-treated cancer cells together with increased intratumoral accumulation of CD103$^+$ DCs at the tumor site after treatment with the CXCR4 antagonist-armed virotherapy, prompted us to explore whether expansion of this subset of DCs with the growth factor FLT3L (12) would contribute to antitumor efficacy, consistent with the ability of tumor-infiltrating CD103$^+$ DCs to prime tumor-specific CD8$^+$ T cells (6). Eight days after viral treatment, the tumor-bearing mice were injected i.p. with FLT3L (5 μg/injection) for four consecutive days and changes in the population of intratumoral myeloid cells were analyzed two days later by flow cytometry. The FLT3L treatment resulted in ~4-fold increases in the accumulation of CD103$^+$ DCs and less than twofold increases in CD11b$^+$ DCs and TAM2 compared to the respective changes mediated by virotherapy treatment alone (**Fig. 4*A***). The increases in CD103$^+$ DCs after FLT3L treatment in peritoneal cavities of virally-treated mice were associated with significant regression of ID8-T tumor compared to tumor-bearing animals that received a monotherapy treatment with OVV-EGFP or OVV-CXCR4-A-Fc (**Fig. 4*B***; $P = 0.04$ and $P = 0.03$), suggesting that the scarcity of CD103$^+$ DCs at the tumor site restricted expansion of tumor-specific CD8$^+$ T cells. To directly address whether the *in vivo* expanded CD103$^+$ DCs were capable of engulfing vaccinia virus-killed ID8-T cancer cells, we isolated CD45$^+$ leukocytes from peritoneal cavities of tumor-bearing mice two days after OVV-EGFP or OVV-CXCR4-A-Fc treatment alone or in combination with the FLT3L growth factor, and cultured them overnight with virally-treated and cell tracker-labeled ID8-T cancer cells. The capture of tumor-associated fluorescent antigens by CD103$^+$ DCs was detected within the myeloid cell compartment by multicolor flow cytometry panel. After subgating of MHCII$^+$F4/80^{lo}cells by the expression of DC-specific marker CD103 and cancer cell tracker, we analyzed the population of double-positive DCs that captured tumor-associated fluorescence. As shown in **Fig. 4*C,D***, approximately 30% of CD103$^+$ DCs in cultures derived from tumor-bearing mice that received OVV-EGFP or OVV-CXCR4-A-Fc virotherapy followed by FLT3L treatment phagocytosed tumor cell debris, which was over twofold higher compared to the DC-mediated phagocytosis in cultures prepared from tumors treated with the respective vaccinia virus only ($P = 0.03$).

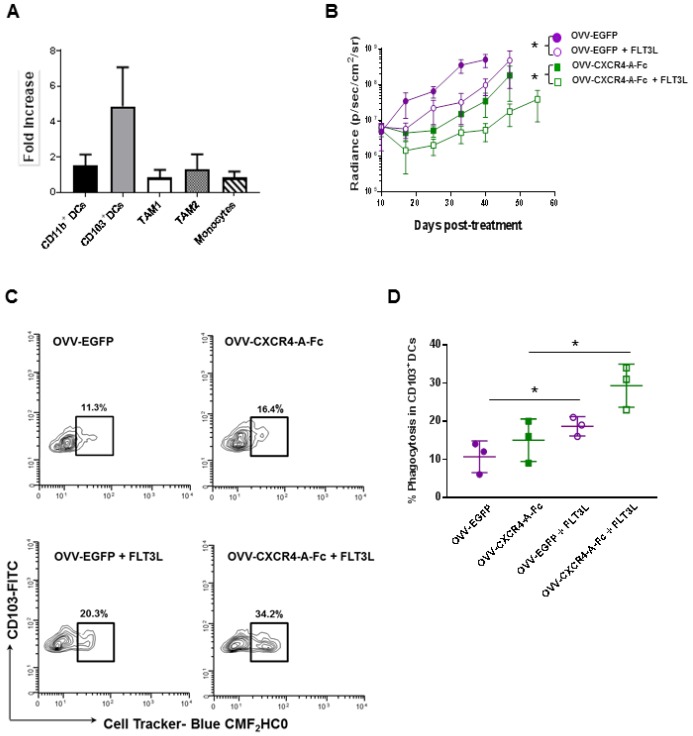

Fig. 4. Expansion of CD103$^+$ DCs at the tumor site by the growth factor FLT3L delays tumor growth and increases phagocytosis of tumor cell debris. (***A***) FLT3L was delivered i.p. to virotherapy-treated ID8-T-bearing mice at 5 μg/injection for 4 consecutive days, beginning on day 8 after virotherapy treatment. The expansion of CD11b$^+$ DCs, CD103$^+$ DCs, TAM1, TAM2, and monocytes in peritoneal washes were analyzed two days after the treatment by flow cytometry. (***B***) Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. (***C***) CD45$^+$ leukocytes isolated from peritoneal cavities of tumor-bearing mice two days after treatments with OVV-EGFP or OVV-CXCR4-A-Fc alone or in combination with FLT3L were cultured with virally-treated and cell tracker-labeled ID8-T cancer cells. After overnight incubation, the capture of tumor-associated fluorescent debris by CD103$^+$ DCs was analyzed by multicolor flow cytometry. One representative experiment of three independent experiments performed is shown. (***D***) Percentages of phagocytosis of virally-treated tumor cell debris by CD103$^+$ DCs are presented as mean ± SD.

Task 3. We have analyzed the ability of the CXCR4 antagonist-armed virus delivered in combination with the growth factor FLT3L to serve as a platform for a therapeutic WT1-specific epitope-based vaccine to enhance antitumor CD8$^+$ T cell responses and overall survival in ID8-T tumor-bearing mice.

Because excess of highly phagocytic macrophages could compete with CD103$^+$ DCs for the TAA availability at the tumor site, we investigated whether this limitation could be overcome by adjuvanted vaccination with a

tumor antigen-based vaccine delivered to peritoneal cavities of ID8-T tumor-bearing mice at the time of expansion of $CD103^+$ DCs.

Progress:

3a. Expansion of $CD103^+$ DCs at the Tumor Site Enhances Tumor Responses to Therapeutic WT1 Peptide Vaccine.

For the immunization, we used a clinically relevant WT1 antigen target that is also expressed on ID8-T cells (3, 13). The WT1-specific peptide (aa 175-202; CRYGPFGPPSQASSGOARMFPNAPYL) containing H2-IA^b-restricted CRYGPFGPPPSQAS and H2-D^b-restricted RMFPNAPYL epitopes was delivered i.p. to ID8-T-bearing mice (50 μg/injection) two days after the last delivery of FLT3L growth factor (**Fig. 5*A***). As an adjuvant, the immunized tumor-bearing mice were treated i.p. with polyriboinosinic:polyribocytidylic acid (polyI:C; 50 μg/injection) that binds to TLR3 on $CD103^+$ DCs (2) and induces type I IFN production and DC maturation (14-16). As shown in **Fig. 5*B***, immunization of OVV-EGFP-treated tumor-bearing mice with the WT1-specific-peptide vaccine delivered after FLT3L treatment significantly extended survival compared with WT1 peptide or FLT3L monotherapy-treated counterparts ($P < 0.001$). The inhibition of tumor growth was more prominent in ID8-T-bearing mice treated with the CXCR4 antagonist-armed virus (**Fig. 5*C***), indicating the ability of the combined OVV-CXCR4-A-Fc treatment and *in situ* DC vaccine with adjuvanted WT1-specific peptide to extend the overall survival. The vaccine-induced tumor regression by the peptide-based immunization strategy was dependent upon expansion of Batf3-driven, $CD103^+$ DCs because the effect was ablated in *Batf3*$^{-/-}$ knockout mice (*SI Appendix*, Fig. S5).

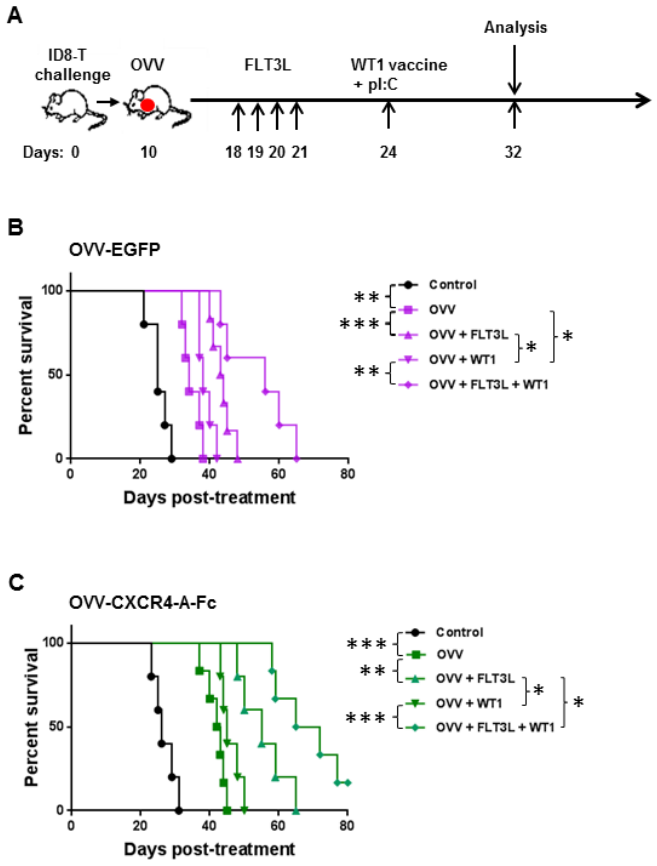

Fig. 5. Expansion of $CD103^+$ DCs at the tumor site enhances tumor responses to therapeutic WT1-specific peptide vaccine. (***A***) Graphical time line of the treatment scheme in ID8-T tumor-bearing mice. C57BL/6 mice (n = 6 - 10) were injected i.p. with 3 x 10^5 ID8-T cells. Treatment with OVV-CXCR4-A-Fc or OVV-Fc (10^8 PFU delivered i.p.) was initiated 10 days later. To expand $CD103^+$ DCs, FLT3L 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) two days after the last FLT3L injection. To promote the maturation of the mobilized $CD103^+$ DCs, the tumor-bearing mice were treated with polyI:C (pI:C; 50 μg/injection) 8 h after WT1 peptide immunization. (***B,C***) Survival was defined as the point at which mice were killed because of extensive tumor burden. Kaplan-Meier survival plots were prepared and significance was determined using the log-rank method. $^{**}P < 0.01$, $^{***}P < 0.001$.

As the accumulated $CD103^+$ DCs at the tumor site are the major component of the establishment of the T cell-inflamed tumor phenotype due to production of the CXCR3-engaging chemokines on effector $CD8^+$ T cells (17), CXCL9 and CXCL10 (18, 19), we analyzed the effect of the single and combined treatment on recruitment of antigen-experienced $CD8^+Ly6C^+$ T cells in ascites or peritoneal washes 7 days after completion of the treatments (20). Immunofluorescence staining performed on single-cell suspensions of $CD45^+CD11b^-$

gated cells with CD8- and Ly6C-specific antibodies revealed ~4-fold expansion of the double-positive cells after virotherapy treatment alone compared to the control mice (**Fig. 6*A,B***). No significant differences in the recruitment of the double-positive $CD8^+Ly6C^+$ T cells were observed between OVV-EGFP- and OVV-CXCR4-A-Fc-treated tumors (**Fig. 6*B,C***).

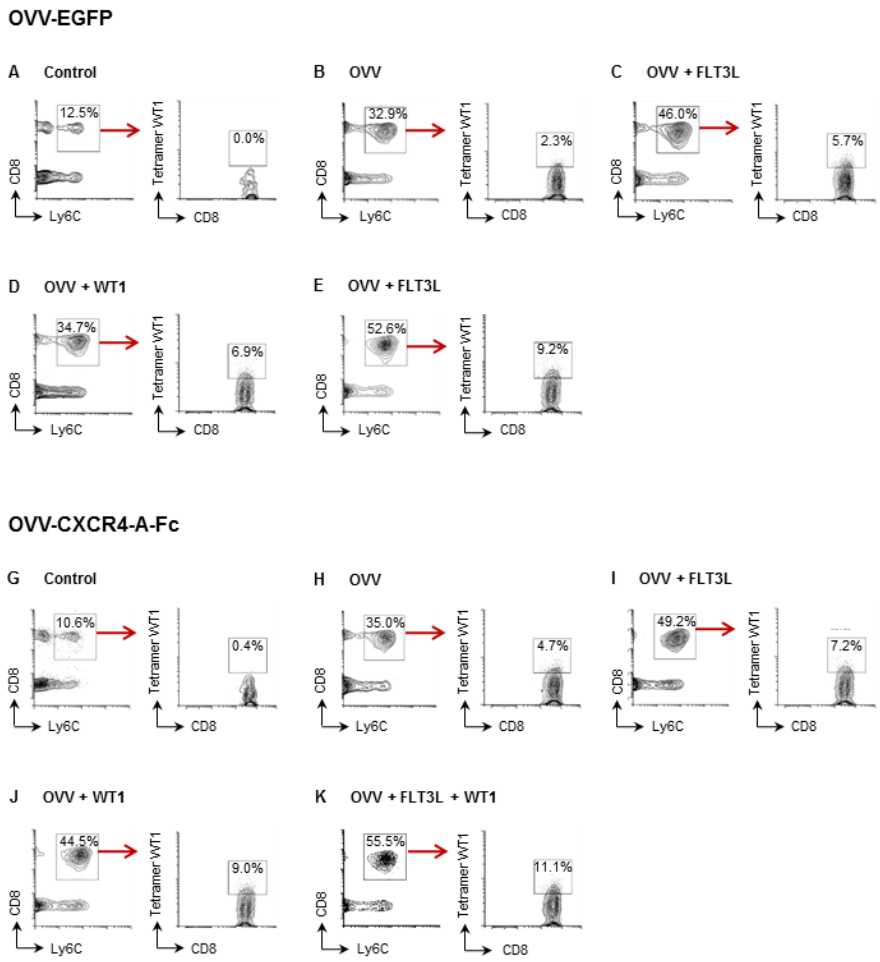

Fig. 6. Evaluation of $CD8^+Ly6C^+$ T cells (*left panel*) and WT1 $tetramer^+$ subset of $CD8^+Ly6C^+$ T cells populations (*right panel*) in ascites-derived tumors or peritoneal washes after combination treatments with: PBS (***A***,***G***); OVV (***B***,***H***); OVV + FLT3L (***C***,***I***); OVV + WT1 (***D***, ***J***); and OVV+ FLT3L + WT1 (***E***, ***K***). Immunofluorescence staining on single-cell suspensions of $CD45^+CD11b^-$ cells with CD8- and Ly6C-specific antibodies was performed 7 days after completion of the treatment depicted in **Fig. 5*A***. One representative experiment of four mice per group performed is shown.

Treatment with FLT3L increased the number of double-positive $CD8^+$ T cells by over 30% ($P < 0.05$), likely due to cytokine-driven expansion and tumor antigen cross-priming reflected by increased percentages of WT1-specific T cells measured with $WT1_{126-134}$/H-$2D^b$ tetramer ($P < 0.05$). Immunization with the WT1 vaccine increased the numbers of WT1 $tetramer^+$ cells in OVV-EGFP- and OVV-CXCR4-A-Fc-treated mice compared to the virally-treated counterparts (Figure 6; $P < 0.01$), though it had a small effect on expansion of the double-positive T cells. On the other hand, the expansion of $CD103^+$ DCs by FLT3L delivery followed by the WT1 peptide vaccine resulted in the highest accumulation of $Ly6C^+CD8^+$ T cells at the tumor site that constituted over 90% of total $CD8^+$ T cell population and contained ~10% WT1 $tetramer^+$ cells. Altogether, these findings indicate the ability of the combined OVV-CXCR4-A-Fc and *in situ* epitope-based vaccine to promote the generation of T cell-inflamed TME with tumor antigen-specific $CD8^+$ T cell responses.

What was accomplished under these goals?

Key Research Accomplishments: We have completed major elements of Aim 2.

During the study period, we have analyzed the effect of targeted delivery of the CXCR4 antagonist by oncolytic vaccinia virus alone or in combination with the growth factor FMS-like tyrosine kinase 3 ligand (FLT3L) on recruitment of DCs to the tumor site and the ability of $CD103^+$ DCs to engulf tumor cell debris as a platform for *in situ* immunization with and adjuvanted WT1-specific peptide-based vaccine. The highlights of this study are as follows:

1). We demonstrated that immunogenic cell death-inducing oncolytic virotherapy with CXCR4-antagonist-expressing oncolytic vaccinia virus 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.

2). We showed that intratumoral delivery of the CXCR4 antagonist-armed virus was more effective in inhibiting tumor growth than the systemic treatment and also had minimal toxicity associated with viral spread and interaction of the antagonist with the CXCR4 receptor expressed by many cell types, including those of the central nervous, gastrointestinal, and immune systems (21).

3). The CXCR4 antagonist expressed as a fusion protein with Fc portion of IgG enhanced the virally-mediated immunogenic cell death and phagocytosis because the CXCR4-A-Fc fusion protein released from virally-infected tumor cells was capable of facilitating phagocytosis of tumor cell material by binding to its cognate receptor on tumor cells and the Fcγ receptors (FcγR) on phagocytes.

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 by injection of FLT3L.

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. Here we showed that expansion of the tumor-residing $CD103^+$ DCs by the growth factor FLT3L after virotherapy treatment served as a platform for a booster immunization with an epitope-based adjuvanted vaccine increasing antitumor immune responses and overall survival. The described immunization strategy uses *in situ* vaccination in which intratumoral administration 'off-the-shelf' products specifically induced and amplified T cell responses to syngeneic tumors and increase overall survival.

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?

Because innate-resistance of non-responding tumors together with immunosuppressive TME negatively affect immunotherapy treatment, the priming with CXCR4 antagonist-armed virotherapy and peptide-vaccine boost strategy reduces tumor load and drives effective antitumor immunity. Although this approach provides a foundation for an efficacious vaccination strategy against ovarian cancer, several questions remain to be addressed in the next reporting period. This includes:

1) The phenotype of WT1 $tetramer^+$ cells within the Ly6C population of $CD8^+$ T cells at the tumor sites needs to be investigated because of the association of Ly6C with central memory $CD44^{hi}CD62L^+$, effector memory $CD44^{hi}CD62L^-$ and, to a lesser extent, the naïve $CD44^{lo}CD62L^+$ T cell subsets (20).

2) The highest accumulation of the double-positive T cells after the combination treatment suggests that infiltration of the antigen-experienced cells in the TME could be driven by the *in situ* vaccination as well as cytokines produced by the expanded $CD103^+$ DCs. This could lead to the generation of T cells subsets with different antitumor efficacy and persistence, which could be evaluated by an adoptive transfer to congenic tumor-bearing mice.

3) Because the differentiation status of T cells affects antitumor efficacy associated with expression of inhibitory receptor programmed death 1 (PD-1) or the programmed death ligand 1 (PD-L1) (22), further analyses are needed to determine whether addition of anti-PD1 or anti-PD-L1 antibody treatment promotes the persistence and effector function of tumor-specific $CD8^+$ T cell immune responses.

4) The key findings obtained in the ID8-T-challenged C57BL/6 mice will be validated using C57BL/6 TgMISIIR-TAg-Low transgenic mice that serve as immunocompetent syngeneic allograft recipients for ovarian MOVCAR cell line (23).

All further studies described in the original application remain essentially unchanged. The results of our studies argue that strategies that reduce tumor load along with the immunosuppressive network in the TME and restore both the recruitment and activation of $CD103^+$ DCs provide foundation for effective *in situ* vaccination with tumor associated antigens and has the potential to transform clinical responses to immune checkpoint inhibitor treatments.

4. IMPACT

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

In this study, we demonstrated that expansion of tumor-residing $CD103^+$ DCs by the growth factor FLT3L after CXCR4 antagonist-armed virotherapy treatment served as a platform for booster immunization with a peptide-based adjuvanted vaccine increasing antitumor immune responses and overall survival. The described immunization strategy uses *in situ* vaccination in which intratumoral administration 'off-the-shelf' products specifically induces and amplifies T cell responses to syngeneic tumors, providing a foundation for development of more efficacious vaccines against ovarian cancer.

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:

Komorowski, M., Gil, M., Mistarz, A., McGray, A. J. R, Opyrchal, Jiang, A., Opyrchal, M., Odunsi, K., and **Kozbor, D.** Intratumoral expansion of $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy augments *in situ* boost with a peptide-based vaccine. Proc. Natl. Acad. Sci, USA, submitted. 2018.

Presentations:

Danuta Kozbor - Invited speaker
"Targeting Drug-resistant Ovarian Cancer with Oncoimmunotherapy"
20th Annual Upstate New York Immunology Conference
The Sagamore Resort and Conference Center
Bolton Landing, NY
October 23-26, 2017
www.amc.edu/nyic/upload/2017-NYIC-Program.pdf

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)

Austin Miller, Ph.D.
Biostatistician
Effort: 0.36 calendar months (no change; in the next reporting period, Dr. Miller's effort will be replaced by Dr. Li Yan from the Department of Biostatistics)

Marcin Komorowski, M.Sc.
Research Affiliate
Effort: 9.6 calendar months (recently resigned from the project and his effort was replaced by Anna Mistarz, M.Sc.)

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:

- Updated Curriculum Vitae
- Supplementary Figures 1-5.
- Manuscript: Komorowski, M., Gil, M., Mistarz, A., McGray, A. J. R, Opyrchal, Jiang, A., Opyrchal, M., Odunsi, K., and **Kozbor, D.** Intratumoral expansion of $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy augments *in situ* boost with a peptide-based vaccine. Proc. Natl. Acad. Sci, USA, submitted. 2018.

10. REFERENCES

1. Broz ML, Binnewies M, Boldajipour B, Nelson AE, Pollack JL, Erle DJ, et al. Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell.* 2014;26(5):638-52.
2. Hashimoto D, Miller J, and Merad M. Dendritic cell and macrophage heterogeneity in vivo. *Immunity.* 2011;35(3):323-35.
3. Gil M, Komorowski MP, Seshadri M, Rokita H, McGray AJ, Opyrchal M, et al. CXCL12/CXCR4 blockade by oncolytic virotherapy inhibits ovarian cancer growth by decreasing immunosuppression and targeting cancer-initiating cells. *J Immunol.* 2014;193(10):5327-37.
4. Gil M, Seshadri M, Komorowski MP, Abrams SI, and Kozbor D. Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(14):E1291-300.
5. Komorowski MP, McGray AR, Kolakowska A, Eng K, Gil M, Opyrchal M, et al. Reprogramming antitumor immunity against chemoresistant ovarian cancer by a CXCR4 antagonist-armed viral oncotherapy. *Molecular Therapy Oncolytics.* 2016;3:16034.
6. Salmon H, Idoyaga J, Rahman A, Leboeuf M, Remark R, Jordan S, et al. Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity.* 2016;44(4):924-38.
7. van Vloten JP, Workenhe ST, Wootton SK, Mossman KL, and Bridle BW. Critical Interactions between Immunogenic Cancer Cell Death, Oncolytic Viruses, and the Immune System Define the Rational Design of Combination Immunotherapies. *J Immunol.* 2018;200(2):450-8.
8. Sistigu A, Yamazaki T, Vacchelli E, Chaba K, Enot DP, Adam J, et al. Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nature Medicine.* 2014;20(11):1301-9.
9. Sukkurwala AQ, Martins I, Wang Y, Schlemmer F, Ruckenstuhl C, Durchschlag M, et al. Immunogenic calreticulin exposure occurs through a phylogenetically conserved stress pathway involving the chemokine CXCL8. *Cell Death and Differentiation.* 2014;21(1):59-68.
10. Garg AD, and Agostinis P. Editorial: Immunogenic Cell Death in Cancer: From Benchside Research to Bedside Reality. *Frontiers in Immunology.* 2016;7:110.
11. Garg AD, Romano E, Rufo N, and Agostinis P. Immunogenic versus tolerogenic phagocytosis during anticancer therapy: mechanisms and clinical translation. *Cell Death and Differentiation.* 2016;23(6):938-51.
12. Liu K, and Nussenzweig MC. Origin and development of dendritic cells. *Immunol Rev.* 2010;234(1):45-54.
13. Hylander B, Repasky E, Shrikant P, Intengan M, Beck A, Driscoll D, et al. Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. *Gynecologic Oncology.* 2006;101(1):12-7.
14. Bogunovic D, Manches O, Godefroy E, Yewdall A, Gallois A, Salazar AM, et al. TLR4 engagement during TLR3-induced proinflammatory signaling in dendritic cells promotes IL-10-mediated suppression of antitumor immunity. *Cancer Research.* 2011;71(16):5467-76.
15. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *The Journal of Experimental Medicine.* 2009;206(7):1589-602.
16. Matsumoto M, and Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev.* 2008;60(7):805-12.
17. Mikucki ME, Fisher DT, Matsuzaki J, Skitzki JJ, Gaulin NB, Muhitch JB, et al. Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nature Communications.* 2015;6:7458.
18. Salerno EP, Olson WC, McSkimming C, Shea S, and Slingluff CL, Jr. T cells in the human metastatic melanoma microenvironment express site-specific homing receptors and retention integrins. *International Journal of Cancer.* 2014;134(3):563-74.
19. Spranger S, Dai D, Horton B, and Gajewski TF. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell.* 2017;31(5):711-23 e4.
20. DeLong JH, Hall AO, Konradt C, Coppock GM, Park J, Harms Pritchard G, et al. Cytokine- and TCR-Mediated Regulation of T Cell Expression of Ly6C and Sca-1. *J Immunol.* 2018;200(5):1761-70.

21. Gupta SK, and Pillarisetti K. Cutting edge: CXCR4-Lo: molecular cloning and functional expression of a novel human CXCR4 splice variant. *J Immunol.* 1999;163(5):2368-72.
22. Seifert AM, Zeng S, Zhang JQ, Kim TS, Cohen NA, Beckman MJ, et al. PD-1/PD-L1 Blockade Enhances T-cell Activity and Antitumor Efficacy of Imatinib in Gastrointestinal Stromal Tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2017;23(2):454-65.
23. Quinn BA, Xiao F, Bickel L, Martin L, Hua X, Klein-Szanto A, et al. Development of a syngeneic mouse model of epithelial ovarian cancer. *J Ovarian Res.* 2010;3:24.

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):

POSITION TITLE: Associate Professor and Associate 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 antitumor activities of immuno-oncolytic viral vectors against primary and metastatic ovarian tumors. We design novel approaches to enhance the antitumor efficacy of oncolytic virotherapy by expressing therapeutic genes and augmenting spread of the virus within tumors in preclinical studies in mice. Specifically, we have evaluated the effect of selective disruption of tumor vasculature by photodynamic therapy (PDT) on the therapeutic activity of systemically administered thymidine kinase (*TK*)- and vaccinia growth factor (*VGF*)-deleted oncolytic vaccinia virus. Using highly metastatic variants of ovarian tumors, we investigate methods to reprogram antitumor immune responses by a CXCR4 antagonist-armed oncolytic vaccinia virus. We anticipate that the oncotherapy-mediated changes in the tumor microenvironment will modulate the interaction between malignant and stromal cells and facilitate induction of protective antitumor immune responses. These studies, if successful, have the potential to be translated into the clinic though collaboration with our clinical investigators and will help in exploring the mechanistic underpinnings of tumor-immune system interactions.

As a postdoctoral fellow at the Wistar Institute in Philadelphia, PA, I gained experience in molecular genetics during the research on the mechanism 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 of disease progression, and induction of HIV-specific immune responses using a recombinant vaccinia virus as a vector for 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 study. 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 Cancer Institute, 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 Cancer Institute, Buffalo, NY
2012 *Ad Hoc* Reviewer; NIH, Cancer Immunopathology and Immunotherapy Study Section

C. Contributions 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.

Gil, M., Bieniasz, M., Wierzbicki, A., Bambach, B., Rokita, H., and Kozbor, D. Targeting a Mimotope Vaccine to Activating Fc gamma Receptors Empower Dendritic Cells to Prime Specific CD8+ T Cell Responses in Tumor-bearing Mice. J. Immunology, 2009, 183:6808-18, PMCID: PMC2805007.

Gil M., Seshadri M., Abrams S. I, Kozbor, D. Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases. Proc. Natl. Acad. Sci, USA, 2013, 110:E1291-E1300, PMCID: PMC3619300.

Gil, M., Komorowski, M., Seshadri, M., Rokita, H., McGray, A.J.R., Opyrchal, M., Odunsi, K., and Kozbor, D. CXCL12/CXCR4 blockade by oncolytic virotherapy inhibits ovarian cancer growth by decreasing immunosuppression and targeting cancer initiating cells. J.Immunol, 2014, 193:5327-5337, PMCID: PMC4225176.

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, 3:16034-16048. PMCID: PMC5155641

McGray, A.J.R., Huang, R.Y., Battaglia, S., Eppolito, C., Miliotto., Stephenson, K., Lugade, A., Webster, G., Lichty, B., 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. Nature Communications, submitted, 2018.

Komorowski, M., Gil, M., Mistarz, A., McGray, A. J. R, Opyrchal, Jiang, A., Opyrchal, M., Odunsi, K., and Kozbor, D. Intratumoral expansion of $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy augments *in situ* boost with a peptide-based vaccine. Proc. Natl. Acad. Sci, USA, submitted. 2018.

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 $V\delta1^+$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 $V\delta1^+$ 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.

Bolesta E., Gzyl, J., Wierzbicki, A., Kmieciak, D., Kowalczyk, A., Srinivasan, A., Kaneko, Y., and Kozbor, D. Clustered Epitopes within Gag-Pol Fusion Protein DNA Vaccine Enhance Immune Responses and Protection against Challenge with Recombinant Vaccinia Viruses Expressing HIV-1 Gag and Pol Antigens. Virology 332:467-479, 2005.

Bolesta, E., Kowalczyk, A., Wierzbicki, A., Eppolito, C., Shrikant, PA. and Kozbor. D. Increased Level and Longevity of Protective Immune Responses Induced by DNA Vaccine Expressing the HIV-1 Env Glycoprotein when Combined with IL-21 and IL-15 Gene Delivery. J. Immunology, 2006, 177: 177-191.

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.

Kozbor, D., Giallongo, A., Sierzega, M.E., Konopka, J.B., Witte, O.N., Showe, L.C., and Croce, C.M. Expression of a translocated c-abl gene in hybrids of mouse fibroblasts and chronic myelogenous leukemia cells. Nature, 319: 331-333, 1986.

Kozbor, D., Moretta, A., Messner, H.A., Moretta., L., and Croce, C.M. Tp44 molecules involved in antigen-independent T cell activation are expressed on human plasma cells. J. Immunol., 138: 4128-4132, 1987.

Kozbor, D., Burioni, R., ar-Rushdi, A., Zmijewski, C., and Croce, C.M. Expression of members of immunoglobulin gene family in somatic cell hybrids between human B-and T-cells. Proc. Natl. Acad. Sci. USA, 84: 4969-4973, 1987.

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.

Kozbor, D., Lagarde, A.E., and Roder, J.C. Human hybridomas constructed with antigen specific EBV-transformed cell lines. Proc. Natl. Acad. Sci, USA 79: 6651-6655, 1982.

Kozbor, D. and Roder, J.C. In vitro stimulated lymphocytes as a source of human hybridomas. Eur. J. Immunol. 14: 23-27, 1983.

Kozbor, D., Tripputi, P., Roder, J.C., and Croce, C.M. A human hybrid myeloma for production of human monoclonal antibodies. J. Immunol. 133(6): 3001-3005, 1984.

Complete List of Published Work in My Bibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/danuta.kozbor.1/bibliography/44160804/public/?sort=date&direction=ascending

SUPPLEMENTARY INFORMATION

CXCR4 Antagonist as an Adjuvant in Immunotherapy of Epithelial Ovarian Cancer

AWARD NUMBER: W81XWH-16-1-0416

PI: Danuta Kozbor, Ph.D.
Department of Immunology
Roswell Park Comprehensive Cancer Center
Buffalo, NY 14263

Supplementary Figures 1-5

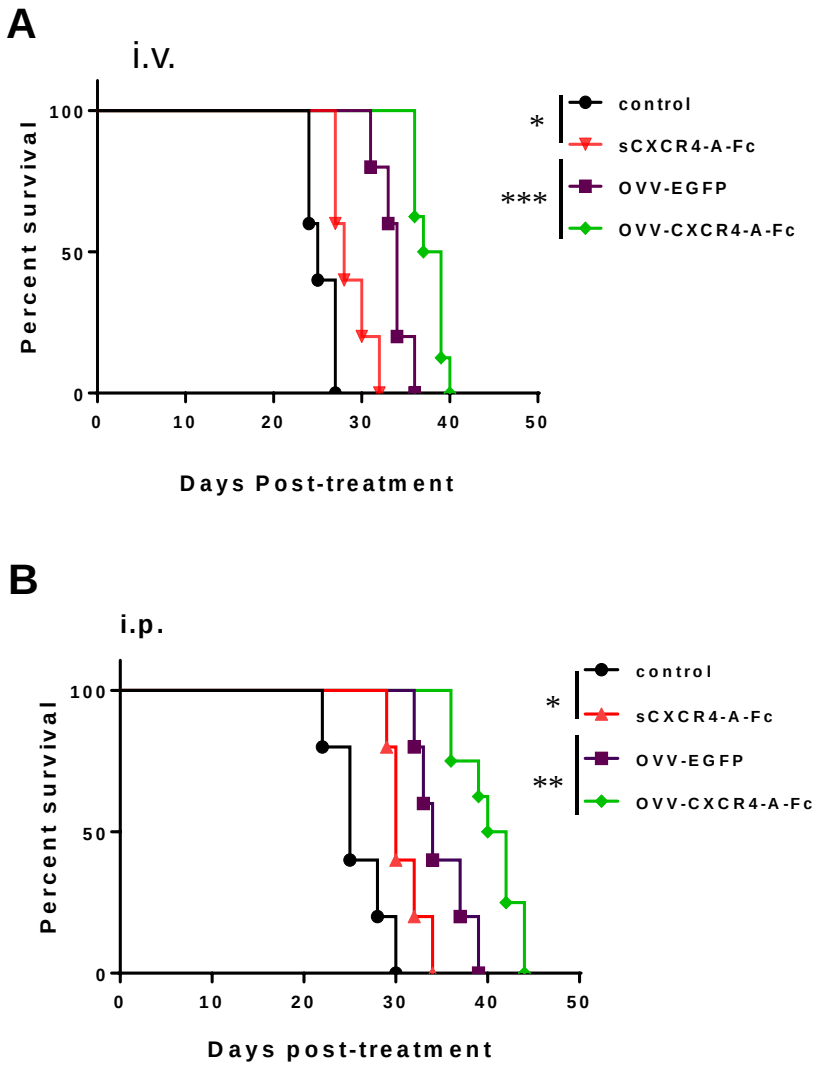

Fig. S1. Effect CXCR4-A-Fc antagonist after i.v. or i.p. delivery on orthotopic ID8-T tumor growth. C57BL/6 female mice (n = 8) were challenged i.p. with ID8-T syngeneic ovarian tumor cells (3x10^5 cells). The tumor-bearing mice were treated with sCXCR4-A-Fc (10 μg/injection), or virally-delivered OVV-CXCR4-A-Fc antagonist (10^8 PFU) injected i.v. (**A**) or i.p. (**B**) 10 days after tumor challenge. The soluble sCXCR4-A-Fc antagonist was delivered daily for 7 days. Control mice were treated with PBS. (**b**) Kaplan-Meier survival plots were prepared and significance was determined using the log-rank method. *P < 0.05, **P < 0.01, ***P < 0.001.

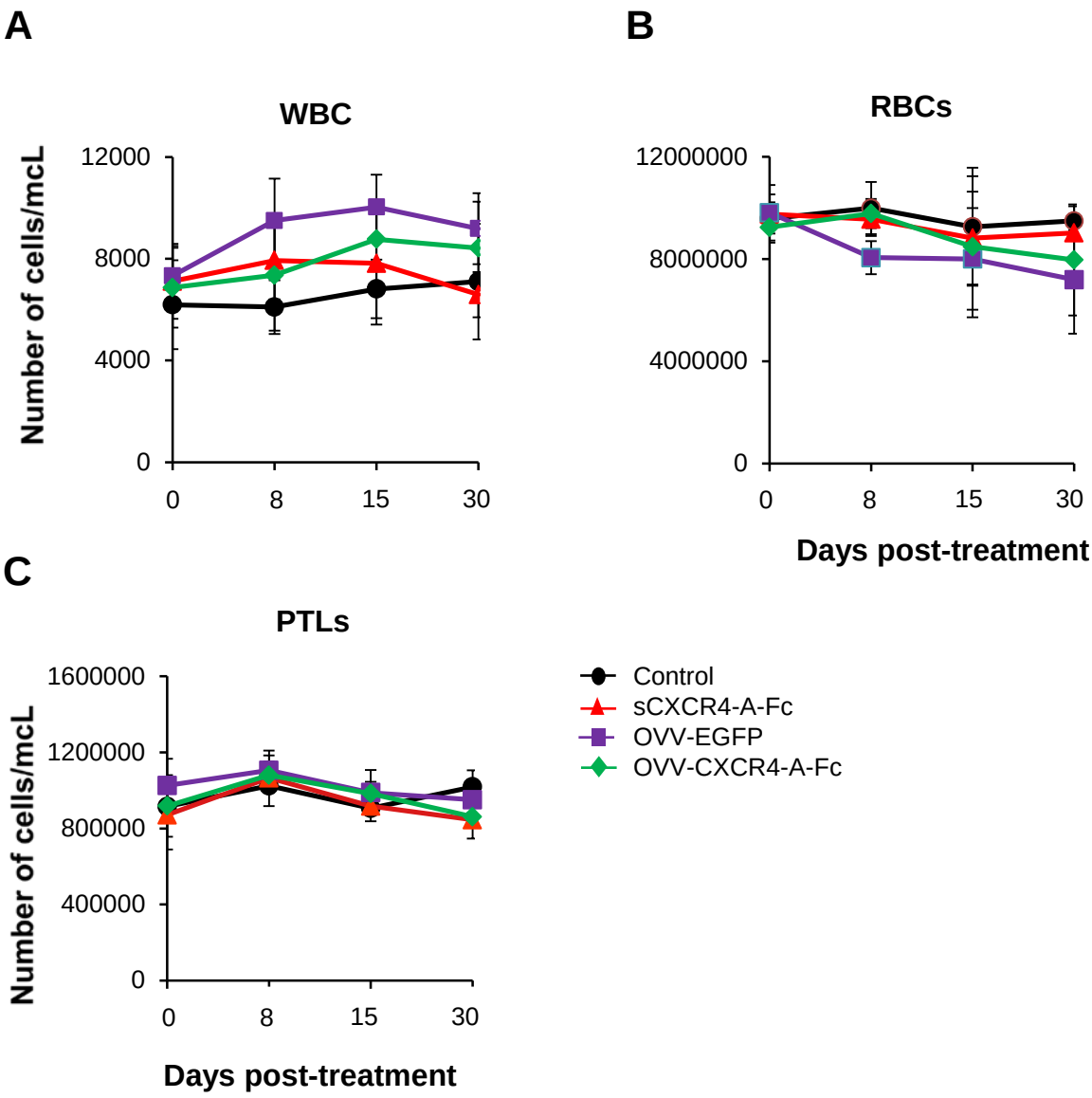

Fig. S2. Cell blood counts (CBCs) after treatment with CXCR4 antagonist-armed oncolytic vaccinia virus or sCXCR4-A-Fc fusion protein delivered i.v. to ID8-T tumor-bearing mice. Mice (n = 5) were bled from the retro-orbital sinus to obtain complete counts of white blood cells (WBCs; ***A)***, red blood cells (RBCs; ***B***), and platelets (PLTs; ***C***) before treatment (day 0) 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.,).

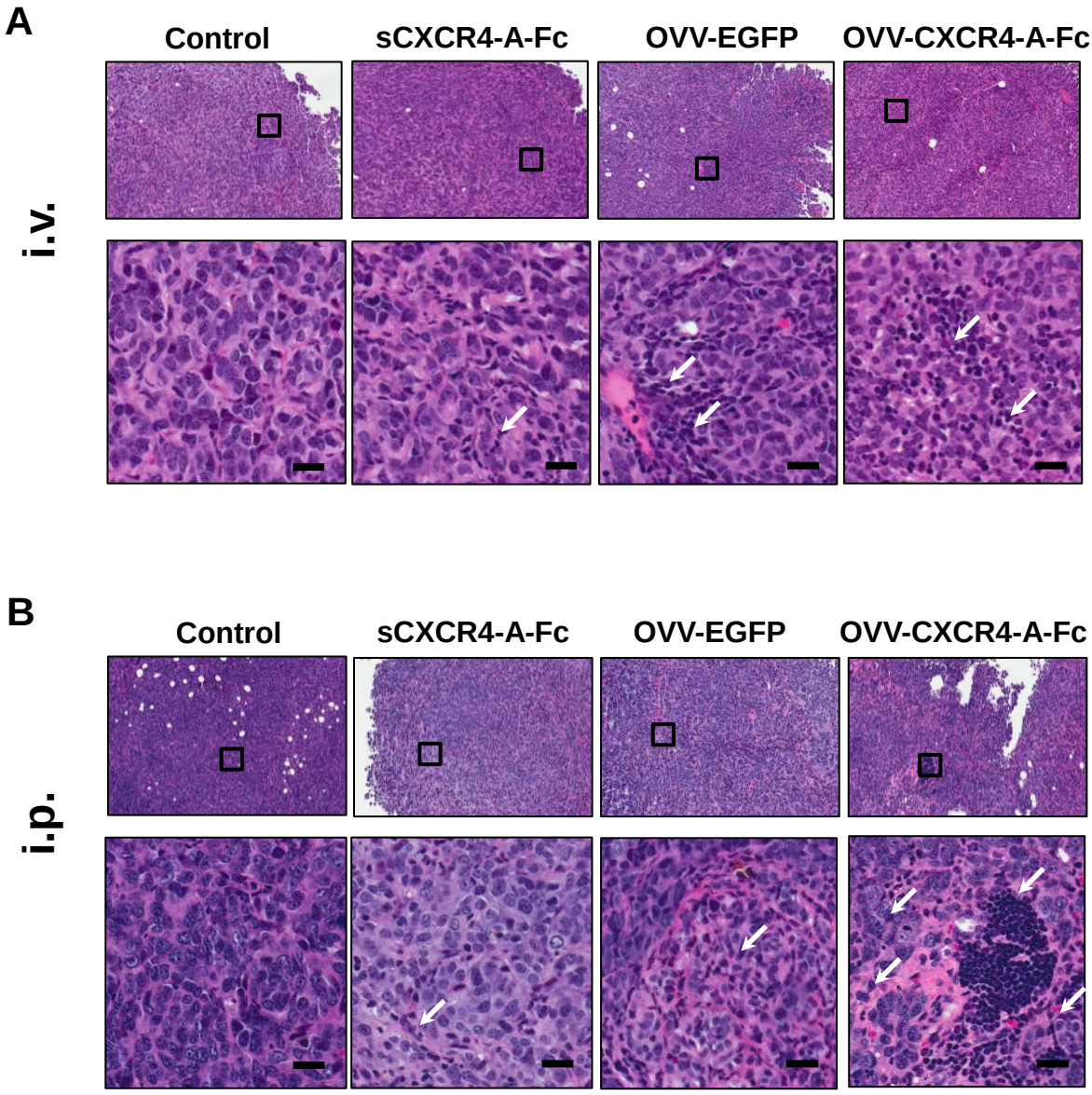

Fig. S3. H&E staining of paraffin sections of omental tumors from mice treated with the CXCR4 antagonist delivered as a soluble protein (CXCR4-A-Fc) or by an oncolytic vaccinia virus. The ID8-T tumor-bearing mice were treated with the CXCR4-A-Fc antagonist, OVV-Fc or OVV-CXCR4-A-Fc injected i.v. (**A**) or i.p. (**B**) 10 days after tumor challenge. Control mice were treated with PBS. Thirty days after each treatment, omental tumors were removed, fixed, paraffin embedded, and sectioned. Five-micrometer-thick sections were stained with H&E (scale bars, 25 μm). Arrows indicate infiltration of leukocytes in tumor tissues.

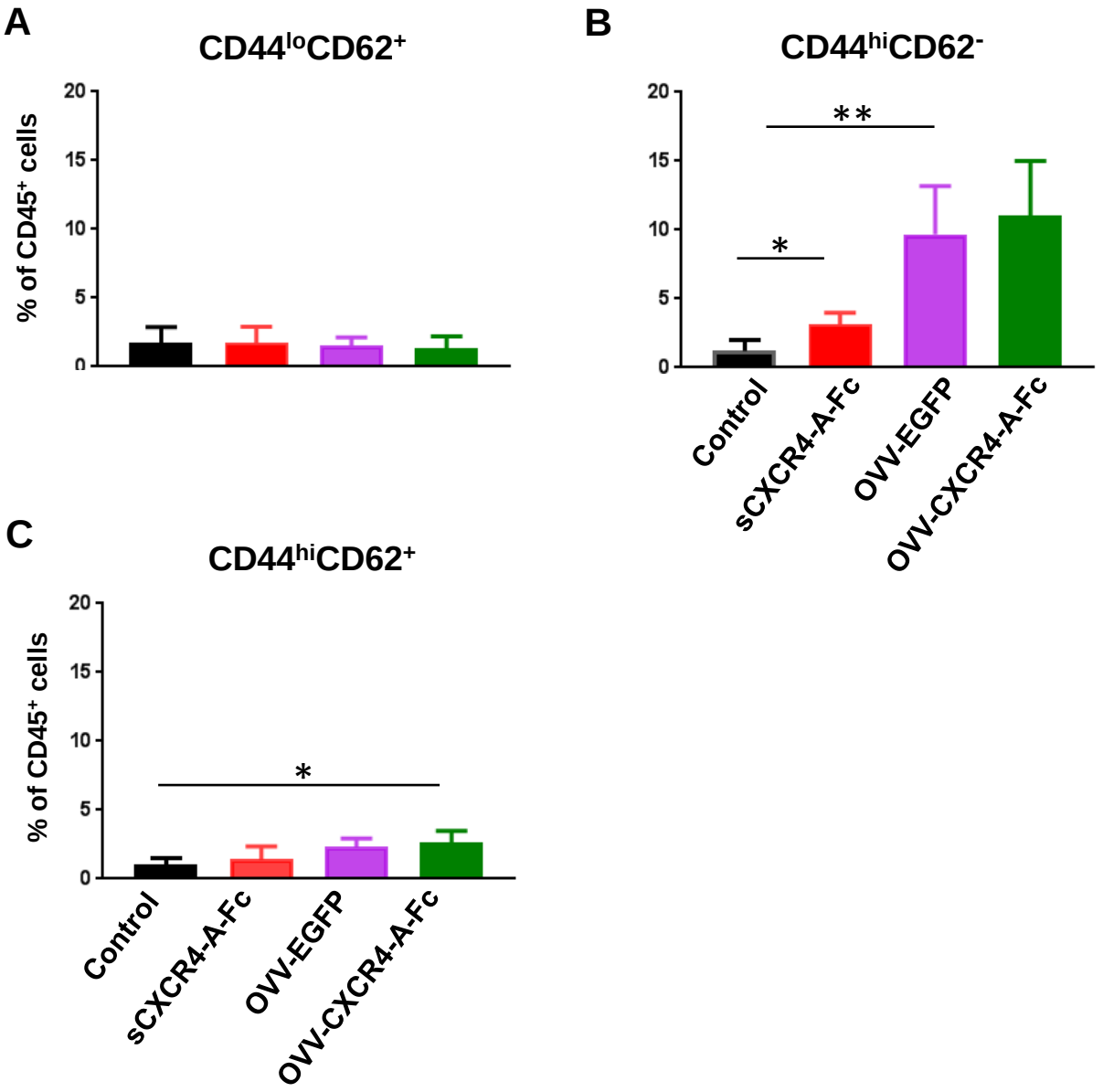

Fig. S4. The effect of the CXCR4 antagonist delivered intratumorally as a soluble fusion protein or by oncolytic vaccinia virus on the phenotype of $CD8^+$ T cells. Phenotypic analyses of naïve $CD44^{lo}CD62L^+$ (**A**), effector $CD44^{hi}iCD62L^-$ (**B**), and memory $CD44^{hi}CD62L^+$ $CD8^+$ T cell infiltrates in ascites-derived tumors or peritoneal washes were performed on CD45-gated cells with mAbs specific for CD44 and CD62L antigens on day 8 after treatment. Background staining was assessed using isotype control antibodies. Results are presented as mean ± SD of five mice per group. $*P < 0.05$, $**P < 0.01$.

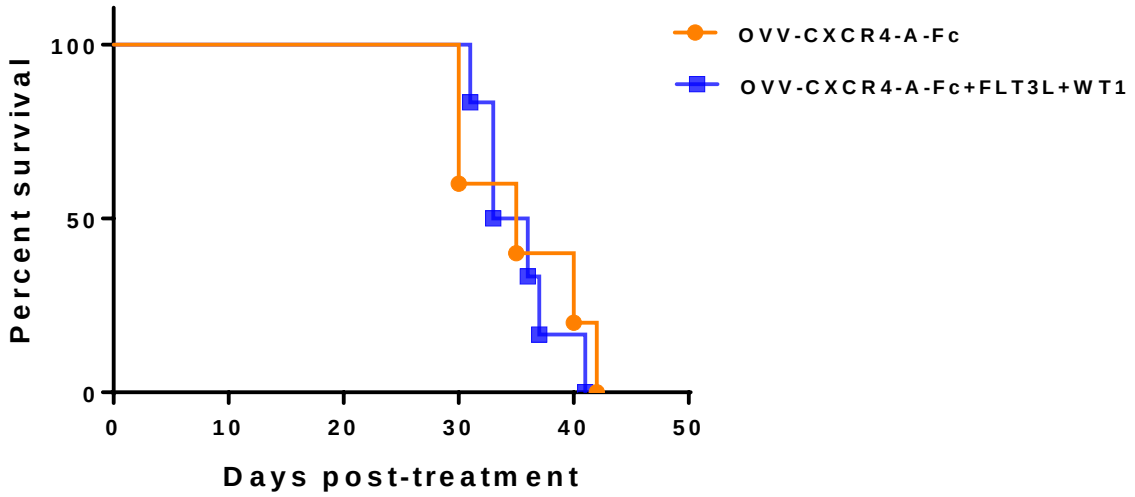

Fig. S5. Inhibition of ID8-T tumor growth in *Batf3*$^{-/-}$ mice by the CXCR4 antagonist-armed oncolytic virotherapy alone or in combination with the WT1 peptide-based vaccine. *Batf3*$^{-/-}$ female mice (n = 5) were challenged i.p. with ID8-T ovarian tumor cells (3x10^5 cells). The tumor-bearing mice were treated with OVV-CXCR4-A-Fc (10^8 PFU) delivered intratumorally 10 days after tumor challenge. For the *in situ* immunization, the growth factor FLT3L was delivered i.p. (5 μg/injection) for four consecutive days beginning on day 8 after virotherapy treatment, followed by the WT1-specific vaccine (50 μg/injection) and pI:C (50 μg/injection) delivered i.p. two days later. Progression of tumor growth was analyzed by bioluminescence. Kaplan-Meier survival plots were prepared and significance was determined using the log-rank method.

Classification: Biological Sciences

Intratumoral expansion of CD103^{+} DCs after CXCR4 antagonist-armed oncovirotherapy augments *in situ* boost with a peptide-based vaccine

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Keywords: CXCR4 antagonist, Oncovirotherapy, *In situ* vaccine

Abstract

Oncolytic viruses are capable of *in situ* vaccination as they induce immunogenic cell death in cancer cells and release tumor associated antigens for priming of tumor-specific $CD8^+$ T cells by tumor-infiltrating $CD103^+$ dendritic cells (DCs). Using a metastatic ID8-T ovarian tumor model in syngeneic mice, we explored whether expansion of $CD103^+$ DCs following a CXCR4 antagonist-armed oncolytic vaccinia virus treatment could augment *in situ* booster immunization with a cancer peptide-based vaccine. We found that intratumoral delivery of the armed virus reduced tumor load and the immunosuppressive network leading to increased infiltration of $CD8^+$ T cells and $CD103^+$ DCs at the tumor site. The latter cells were capable of phagocytic clearance of cellular debris from virally-infected ID8-T tumor. Expansion of the tumor-residing $CD103^+$ DC population by injecting the growth factor FLT3L provided a platform for boost with a peptide-based adjuvanted vaccine. Intraperitoneal vaccination with the Wilms' tumor antigen 1 (WT1)-specific T helper and CTL epitopes together with Toll-like receptor 3 (TLR3)-specific agonist, polyI:C, increased the antitumor efficacy. The treatment-mediated inhibition of tumor growth was associated with increased intratumoral recruitment of WT1 $tetramer^+CD8^+$ T cells that were harbored within the antigen-experienced $Ly6C^+$ T cell population. Our results revealed that expansion of intratumoral $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy treatment enhances *in situ* booster immunization with an adjuvanted tumor-specific epitope-based vaccine and elicits potent and sustained anti-tumor immunity at the tumor site, thus achieving better therapeutic efficacy.

Significance Statement

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 polytope vaccines. Here we showed that expansion of the tumor-residing $CD103^+$ DCs by the growth factor FLT3L after CXCR4 antagonist-armed virotherapy treatment served as a platform for a booster immunization with an epitope-based adjuvanted vaccine increasing antitumor immune responses and overall survival. The described immunization strategy uses *in situ* vaccination in which intratumoral administration “off-the-shelf’ products specifically induced and amplified T cell responses to syngeneic tumors.

Introduction

An effective *in situ* vaccination uses intratumoral immunomodulation to induce immunogenic tumor cell death (ICD) to release tumor-associated antigens (TAAs) from tumor cells, inhibit multiple levels of immunosuppression in the tumor microenvironment (TME), and increase intratumoral dendritic cell (DC) populations capable of cross-presentation of TAAs to antigen-specific T cells (1). Alongside traditional ICD inducers like select chemotherapies and radiation (2), oncolytic viruses (OVs) have emerged as new members of this class of drugs (3-5). Oncolytic virotherapy has been recognized as another form of immunotherapy (3, 4, 6) with a herpes simplex virus expressing GM-CSF recently approved by the Food and Drug administration (7) and other vectors, including vaccinia virus, undergoing extensive evaluation in multiple preclinical and clinical trials (8-13). However, the therapeutic efficacy of OVs in patients has so far been limited, emphasizing the need for combination therapies with classical ICD-inducing chemotherapeutics (10), checkpoint inhibitors to combat PD-1/PDL-1 expression (14), and adjuvanted vaccines to elicit efficient anti-tumor immunity (15). We have demonstrated that innate resistance properties of non-responding ovarian tumors together with the tumor immunosuppressive network could be overcome by oncolytic vaccinia virus (OVV)-delivered CXCR4 antagonist, which was particularly effective in combination with doxorubicin-mediated killing (10). We focused on the CXCR4/CXCL12 axis because of its multiple adverse effects on progression of epithelial ovarian cancer (EOC), including cancer cell migration, invasion, stimulation of VEGF-mediated angiogenesis (16) and intratumoral recruitment of endothelial progenitor cells (17, 18), suppressive $CD11b^{+}Gr1^{+}$ myeloid cells (19-21), and Tregs (21, 22). Therefore, modulation of the CXCL12/CXCR4 axis in EOC could impact multiple aspects of tumor pathogenesis including innate and adaptive immune mechanisms of tumor destruction.

This process however is largely dependent on a delivery mode of the CXCR4 antagonist-armed virus, which affects both viral titers and concentration of the CXCR4 antagonist in tumor versus systemic tissues that may affect the induction of antitumor immune responses though inflammatory clearance of dying cancer cells by APCs at the tumor site.

Through their ability to present TAAs together with costimulatory or inhibitory signals and production of an array of cytokines and chemokines, tumor-infiltrating APCs are best poised to regulate antitumor immune responses (23). Among the tumor-residing APCs, which comprise DCs, macrophages, monocytes, and monocyte-derived cells (24), DCs in mice consist of two functionally specialized subsets: the $CD103^+$ DCs, which excel in the priming and cross-presentation of TAAs to $CD8^+$ T cells, and $CD11b^+$ DCs, which are more potent at driving $CD4^+$ helper T cell responses (24). Analogous counterparts to these populations include in humans include $CD141^+$ and $CD1c^+$ DCs, respectively (25). Accumulating evicence suggess 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 (26). On the other hand, defective recruitment and activation of $CD103^+$ DCs leads to reduced cross-priming of $CD8^+$ T cells and non-T-cell-inflamed tumors (27, 28). Using a ovarian ID8-T tumor model in syngeneic mice, we investigated the effect of targeted delivery of the CXCR4 antagonist by vaccinia virus alone or in combination with growth factor FMS-like tyrosine kinase 3 ligand (FLT3L) on intratumoral recruitment of DCs and their ability to engulf tumor cell debris as a platform for *in situ* boost with an adjuvanted WT1-specific peptide vaccine

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The CXCR4 antagonist, expressed in the context of the murine Fc fragment of IgG2a by oncolytic vaccinia virus (OVV-CXCR4-A-Fc), was delivered intraperitoneally (i.v.) or intravenously (i.p.) to syngeneic mice after orthotopic challenge with metastatic ID8-T ovarian tumor. We found that intraperitoneal delivery of OVV-CXCR-A-Fc was more efficacious in inhibiting tumor growth compared to treatment with the soluble CXCR4-A-Fc (sCXCR4-A-Fc) antagonist or a systemic injection of the armed-virus due to high accumulation of the fusion protein in tumor rather than systemic tissues. Targeting syngeneic tumors with this regimen increased intratumoral infiltration of $CD8^+$ T cells and $CD103^+$ DCs, which was further enhanced through reduction of the immunosuppressive network by virally-delivered CXCR4 antagonist. Subsequent expansion of tumor-residing $CD103^+$ DCs by injection of the growth factor FLT3L and intraperitoneal vaccination with the WT1-specific peptide followed by polyriboinosinic:polyribocytidylic acid (polyI:C) treatment increased efficacy of the treatment. The inhibition of tumor growth was associated with the increased accumulation of $CD8^+Ly6C^+$ T cells at tumor sites characterized by high frequencies of WT1 $tetramer^+$ cells. Altogether, these results demonstrated that expansion of intratumoral $CD103^+$ DCs after CXCR4 antagonist-armed oncovirotherapy treatment represents a viable approach for *in situ* therapeutic vaccine to effectively boost antitumor immune responses.

Results

Accumulation of the CXCR4-A-Fc Antagonist in Tumor Tissues after Systemic or i.p. Delivery and its Effect on Inhibition of Tumor Growth.

Using a highly metastatic murine epithelial ovarian cancer cell line ID8-T growing orthotopically in syngeneic C57BL/6 mice, we have analyzed tumor regression after systemic or intratumoral injection of soluble and virally-delivered CXCR4-A-Fc fusion protein. The oncovirotherapy treatment was initiated 10 days after tumor challenge and consisted of a single injection (10^8 PFU/mouse) of OVV-CXCR4-A-Fc or control virus expressing the enhanced green fluorescence protein (OVV-EGFP). To determine the contribution of the antagonist alone to tumor regression, additional groups of tumor-bearing mice were treated for 7 days with the sCXCR4-A-Fc fusion protein (10 μg/injection) or were injected with PBS (control group). Progression of tumor growth, quantified by bioluminescence imaging, revealed rapid tumor cell dissemination in untreated, control mice (**Fig. 1*A***) that were killed within 4 weeks after tumor challenge (*SI Appendix* Fig. S1*A,B*). Systemic delivery of OVV-CXCR4-A-Fc reduced tumor progression and significantly extended survival compared with control mice ($P < 0.0001$) or animals treated with OVV-EGFP ($P = 0.002$; Fig. S1*A*). On the other hand, the systemic delivery of sCXCR4-A-Fc was not very effective in controlling tumor spread and extended survival by ~1 week compared with control mice. The study revealed that antitumor effects of the virotherapy or soluble antagonist were more pronounced after intratumoral delivery (**Fig. 1*B***). Among the treatment approaches, the best efficacy was achieved with the armed virus delivered i.p. The OVV-CXCR4-A-Fc treatment resulted in ~4-wk tumor regression after which period tumor growth continued at a rate similar to that in control mice, extending the survival by ~2 weeks compared to mice treated with sCXCR4-A-Fc ($P < 0.001$; Fig. S1*B*) or one week compared to the OVV-

EGFP-treated counterparts. Histology performed on formalin-fixed omental tumor tissue samples obtained thirty days after each treatment revealed infiltration of leukocytes after OVV-EGFP or OVV-CXCR4-A-Fc treatment. This contrasted with the poorly inflamed tumors in control or sCXCR4-A-Fc-treted mice (*SI Appendix*, Fig. S2*A,B*), indicating that the increased inflammation at the tumor site was largely induced by the viral treatment. The intratumoral delivery of the antagonist either by the virus or in a soluble form resulted in higher concentrations of the sCXCR4-A-Fc fusion protein in the tumor compared with those measured after the systemic delivery ($P < 0.001$), and almost background levels of the antagonist in sera or other organs (**Fig. 1*C,D***). On the other hand, similar levels of the sCXCR4-A-Fc antagonist were detected in tumor tissues, sera and lymphoid organs after the systemic delivery. The higher concentrations of the antagonist in systemic tissues contributed to increased numbers of leukocytes in the peripheral blood by ~10% on days 8 and 15 before returning to the baseline on day 30, and had no effect on red blood cell and platelet counts (*SI Appendix*, Fig. S3A-*C*).

CXCR4 Antagonist Inhibits the Immunosuppressive Network and Enhances Intratumoral Infiltration of $CD8^+$ T cells and $CD11b^+$ Myeloid Cells.

The reduced antitumor efficacy of the control virus compared to that mediated by the CXCR4 antagonist-armed vaccinia after i.p. delivery, despite similar numbers of tumor-infiltrating $CD45^+$ leukocytes (**Fig. 2*A***), emphasized the importance of blocking the CXCL12/CXCR4 signaling axis to inhibit the immunosuppressive network in the TME. We next investigated the effect of the CXCR4 antagonist delivered as a soluble protein or secreted from virally-infected tumor cells on intratumoral accumulation of granulocytic myeloid-derived suppressor cells (G-MDSCs), plasmacytoid DCs (pDCs), and T regulatory cells (Tregs). Flow cytometry analysis

performed on day 8 after completion of the treatment reveal that i.p. delivery of the sCXCR4-A-Fc antagonist resulted in significant reductions of intratumoral infiltration of G-MDSCs ($CD11b^{+}Ly6C^{low}Ly6G^{high}$) and pDCs ($B220^{high}Ly6C^{high}CD11c^{low}$) compared to control mice (**Fig. 2*B,C***; $P = 0.003$ and 0.05, respectively). A similar profile of treatment-induced responses was measured for the tumor-infiltrating $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs although the changes did not reach a significant level (**Fig. 2*D***). Delivering the antagonist to the tumor-bearing mice by vaccinia virus further decreased accumulation of the immunosuppressive elements compared to the treatment with the soluble antagonist or control virus (**Fig. 2*E***). These changes contributed to significantly higher accumulation of both $CD4^{+}$ and $CD8^{+}$ TILs in virally-treated mice compared with control animals (**Fig. 2*E,F***; $P = 0.01$ and $P = 0.004$, respectively), predominantly with the effector memory phenotype ($CD44^{hi}CD62L^{-}$; *SI Appendix*, Fig. S4*A*, *B*). The increased percentages of TILs after oncovirotherapy treatment was also associated with higher infiltration tumor-associated macrophages (TAMs) and DCs. To characterize their diversity, we profiled the tumor-infiltrating $CD45^{+}$ compartment using multi-color flow cytometry and progressive gating strategy (29). Subgating all $CD45^{+}$ hematopoietic cells by the myeloid-specific marker CD11b that were Ly6C-negative allowed removal of neutrophils ($CD11b^{+}Ly6C^{lo}$) and $CD11b^{+}$ $Ly6C^{high}$ monocytes. Within the $CD11b^{+}MHCII^{+}$ population, TAMs were distinguished from DCs based on high F4/80 expression and low CD24 levels. The flow cytometry analysis revealed that the control and armed-virus viruses significantly increased intraperitoneal recruitment of $CD11b^{hi}CD11c^{lo}$ TAM1 and $CD11b^{lo}CD11c^{hi}$ TAM2 compared to the untreated or sCXCR4-A-Fc-treated mice (**Fig. 2*G,H***; $P < 0.03$). Parsing of the $F4/80^{lo}CD24^{hi}$ compartment revealed two populations of DCs based on differential expression of CD11b and CD103, similar to observations made in healthy peripheral tissues (30). As shown in

Fig. 2*I,J*, expression of the CXCR4 antagonist in the TME in virally-treated tumors had no effect on infiltration of $CD11b^+$ DCs but increased accumulation of $CD103^+$ DCs compared to OVV-EGFP-treated tumors counterparts ($P = 0.04$), whereas the soluble CXCR4 antagonist had no significant effect on intratumoral accumulation of either TAMs or DCs compared to control tumors.

Expansion of $CD103^+$ DCs at the Tumor Site by Growth Factor FLT3L Increased Phagocytosis of Tumor Cell Debris by $CD103^+$ DCs.

In view of the previously demonstrated induction of ICD by vaccinia virus in cancer cells (8-10) and ability of $CD103^+$ DCs to transport intact TAAs to the tumor-draining lymph node (23), the increased intratumoral infiltration of $CD103^+$ DCs together with lower accumulation of the immunosuppressive elements in OVV-CXCR4-A-Fc-treated tumors compared to those receiving the control virus could contribute to the improved immunogenic potential of the armed virotherapy (31). In addition, the soluble CXCR4 antagonist secreted from virally-infected cells could augment vaccinia-mediated killing of uninfected tumor cells by binding to CXCR4-expressing ID8-T cells and inducing apoptosis. To investigate this hypothesis, we first analyzed the induction of apoptosis and necrosis in 24-h cultures of ID8-T cancer cells treated with 10 μg/ml of sCXCR4-A-Fc, a concentration of the antagonist that accumulates in supernatants OVV-CXCR4-A-Fc-infected cultures. Additional conditions included tumor cells infected with OVV-Fc or OVV-CXCR4 at multiplicity of infection (MOI) of 1. The induction of apoptosis/necrosis analyzed by flow cytometry with Annexin V-FITC and LIVE/DEAD fixable violet revealed that while sCXCR4-A-Fc antagonist induced apoptosis in ~15% of cells, the OVV-CXCR4-A-Fc treatment increased the number of apoptotic cells apoptosis to ~45%, which

was significantly higher compared to those in cultures treated with the control virus (**Fig. 3*A***; $P < 0.001$). The treatment-induced changes in apoptotic/necrotic cells also affected the engulfment of tumor cell debris by DCs. As shown in **Fig. 3*B***, phagocytosis of OVV-CXCR4-A-Fc-treated ID8-T cells by $CD11c^+$ BM-derived DCs was more effective compared to the soluble antagonist- and OVV-Fc-treated culture ($P < 0.0001$ and $P = 0.02$, respectively), suggesting that the CXCR4-A-Fc fusion protein released for the virally-infected tumor cells directly augmented the antitumor immune responses induced by the armed vaccinia virus in the tumor-bearing mice because immunogenic phagocytosis is stringently required for mounting immune response against dying tumor cells *in vivo* (31-35).

Results of the *in vitro* analysis of phagocytosis of virally-treated cancer cells together with increased intratumoral accumulation of $CD103^+$ DCs at the tumor site after treatment with the CXCR4 antagonist-armed virotherapy, prompted us to explore whether expansion of this subset of DCs with the growth factor FLT3L (36) contributes to antitumor efficacy, consistent with the ability of tumor-infiltrating $CD103^+$ DCs to prime tumor-specific $CD8^+$ T cells (23). Eight days after viral treatment, the tumor-bearing mice were injected i.p. with FLT3L (5 μg/injection) for four consecutive days and changes in the population of intratumoral myeloid cells were analyzed two days later by flow cytometry. The FLT3L treatment resulted in ~4-fold increases in the accumulation of $CD103^+$ DCs and less than twofold increases in $CD11b^+$ DCs and TAM2 compared to the respective changes mediated by virotherapy treatment alone (**Fig. 4*A***). The increases in $CD103^+$ DCs after FLT3L treatment in peritoneal cavities of virally-treated mice were associated with significant regression of ID8-T tumor compared to tumor-bearing animals that received a monotherapy with OVV-EGFP and OVV-CXCR4-A-Fc (**Fig. 4*B***; $P = 0.04$ and P

= 0.03, respectively), suggesting that the scarcity of $CD103^+$ DCs at the tumor site restricted expansion of tumor-specific $CD8^+$ T cells after virotherapy treatment. To directly address whether the *in vivo* expanded $CD103^+$ DCs were capable of engulfing vaccinia virus-killed ID8-T cancer cells, we isolated $CD45^+$ leukocytes from peritoneal cavities of tumor-bearing mice two days after treatments with OVV-EGFP or OVV-CXCR4-A-Fc alone or combined with the FLT3L growth factor, and cultured them overnight with virally-treated and cell tracker-labeled ID8-T cancer cells. The capture of tumor-associated fluorescent antigens by $CD103^+$ DCs was captured within the myeloid cell compartment by multicolor flow cytometry panel. After subgating of $MHCII^+F4/80^{lo}$cells by the expression of DC-specific marker CD103 and cancer cell tracker, we analyzed the population of double-positive DCs that captured tumor-associated fluorescence. As shown in **Fig. 4*C,D***, approximately 30% of $CD103^+$ DCs in cultures derived from tumor-bearing mice that received OVV-EGFP or OVV-CXCR4-A-Fc virotherapy followed by FLT3L treatment phagocytosed tumor cell debris, which was over twofold higher compared to the DC-mediated phagocytosis in cultures prepared from tumors treated with the respective vaccinia virus only (P = 0.03).

A Prime-boost Strategy with Oncovirotherapy and WT1-specific Epitope-based Vaccine Enhances Antitumor $CD8^+$ T Cell Responses and Overall Survival.

Because excess of highly phagocytic macrophages could compete with $CD103^+$ DCs for the TAA availability at the tumor site, we investigated whether this limitation could be overcome by adjuvanted vaccination with a tumor antigen-based vaccine delivered to ID8-T tumor-bearing mice at the time of expansion of $CD103^+$ DCs. For the immunization, we used a clinically relevant WT1 antigen target that is also expressed on ID8-T cells (8, 37). The WT1-specific

peptide (aa 175-202; CRYGPFGPPSQASSGOARMFPNAPYL) containing H2-IA^b-restricted CRYGPFGPPPSQAS and H2-D^b-restricted RMFPNAPYL epitopes was delivered i.p. to ID8-T-bearing mice (50 μg/injection) two days after the last delivery of FLT3L growth factor (**Fig. 5*A***). As an adjuvant, the immunized tumor-bearing mice were treated i.p. with polyriboinosinic:polyribocytidylic acid (polyI:C; 50 μg/injection) that binds to TLR3 on CD103$^+$ DCs (30) and induces type I IFN production and DC maturation via the adaptor protein TIR-domain-containing adaptor-inducing IFN-β (TRIF) (38-40). As shown in **Fig. 5*B***, immunization of OVV-EGFP-treated tumor-bearing mice with the WT1-specific-peptide vaccine delivered after FLT3L treatment significantly extended survival compared with WT1 peptide or FLT3L monotherapy-treated counterparts ($P < 0.001$). The inhibition of tumor growth was more prominent in ID8-T-bearing mice treated with the CXCR4 antagonist-armed virus (**Fig. 5*C***), indicating the ability of the combined OVV-CXCR4-A-Fc treatment and *in situ* DC vaccine with adjuvanted WT1-specific peptide to extend the overall survival. It is also noteworthy that the vaccine-induced tumor regression was dependent upon expansion of Batf3-driven DCs because the effect was ablated in *Batf3*$^{-/-}$ mice (*SI Appendix*, Fig. S5).

As the accumulated CD103$^+$ DCs at the tumor site are the major component of the establishment of the T cell-inflamed tumor phenotype due to production of the CXCR3-engaging chemokines on effector CD8$^+$ T cells (41), CXCL9 and CXCL10 (28, 42), we analyzed the effect of the single and combined treatment on recruitment of antigen-experienced CD8$^+$Ly6C$^+$ T cells in ascites or peritoneal washes 7 days after completion of the treatments (43). Immunofluorescence staining performed on single-cell suspensions of CD45$^+$CD11b$^-$ gated cells with CD8- and Ly6C-specific antibodies revealed ~4-fold expansion of the double-positive cells after

virotherapy treatment alone compared to the control mice (**Fig. 6*A,B***). No significant differences in the recruitment of the double-positive $CD8^+Ly6C^+$ T cells were observed between OVV-EGFP- and OVV-CXCR4-A-Fc-treated tumors (**Fig. 6*B,C***). Treatment with FLT3L increased the number of double-positive $CD8^+$ T cells by over 30% ($P < 0.05$), likely due to cytokine-driven expansion and cross-priming reflected by increased percentages of WT1-specific T cells measured with $WT1_{126\text{-}134}$/H-$2D^b$ tetramer ($P < 0.05$). Immunization with the WT1 vaccine increased the numbers of WT1 $tetramer^+$ cells in OVV-EGFP- and OVV-CXCR4-A-Fc-treated mice compared to the virally-treated counterparts (Figure 6; $P < 0.01$), though it had a small effect on expansion of the double-positive T cells. On the other hand, the expansion of $CD103^+$ DCs by FLT3L delivery followed by the WT1 peptide vaccine resulted in the highest accumulation of $Ly6C^+CD8^+$ T cells at the tumor site that constituted over 90% of total $CD8^+$ T cell population and contained ~10% WT1 $tetramer^+$ cells. Altogether, these findings indicate the ability of the combined OVV-CXCR4-A-Fc and *in situ* epitope-based vaccine to promote the generation of T cell-inflamed TME with tumor antigen-specific $CD8^+$ T cell responses.

Discussion

As the field of cancer therapy continues to incorporate immunotherapy as an integral treatment strategy, developing approaches that potentiate the induction of ICD and overcome non-T-cell inflamed tumors will be critical for the treatment efficacy. We demonstrated that the ICD-inducing vaccinia virus-armed with the CXCR4 antagonist was able to act as *in situ* vaccines in breast and ovarian tumor models and generate antitumor immune responses that cause regression of metastatic tumors (8-10). Here, we showed that intratumoral delivery of the CXCR4 antagonist-armed virus was more effective in inhibiting tumor growth than the systemic treatment and also minimized toxicity associated with viral spread and interaction of the antagonist with CXCR4 expressed by many cell types, including those of the central nervous, gastrointestinal, and immune systems (44). By inducing immunogenic tumor cell death and antigen release at the tumor site by the virus, the *in situ* vaccination is personalized and can be further boosted with tumor antigen polytope vaccines (45-47). In fact, subsequent expansion of $CD103^+$ DC population at the tumor site by injection of FLT3L served as a platform for boost with the adjuvanted WT1-specific polytope increasing antitumor immune responses and overall survival. Results of these experiments demonstrate that the virotherapy-prime and polytope-boost *in situ* approach eliminates the need for *ex vivo* production of individualized whole tumor cell vaccines and can be used with the 'off-the shelf' products for immunization. Furthermore, injectable immunomodulators such as TLR agonists can be mass-produced, are not resource intensive and are therefore practical to optimize in iterative patient cohorts. Altogether, we have described a strategy using '*in situ* vaccination' in which intratumoral administration 'off-the-shelf' products specifically induced and amplified T cell responses to syngeneic tumors.

In this setting, the virotherapy-generated an immunogenic tumor cell cargo for loading DC vaccines can be further improved by combining it with ICD-inducing chemotherapeutic agents, such as doxorubicin (10), or immune checkpoint inhibitors (34, 35). We also found that the virus armed with the CXCR4-A-Fc fusion protein adds in induction of phagocytosis as the antagonist released from virally-infected tumor cells induced apoptosis after biding to CXCR4 on tumor cells and interacting with the Fcγ receptors (FcγR) on phagocytes. This observation is consistent with our previous report on the ability of the CXCR4-A-Fc fusion protein to eliminate tumor cells through ADCC mechanism (8, 9), indicating that antibody-dependent cell death can help to achieve the desirable 'off-target' induction of antitumor immunity (34, 35). This might be particularly relevant in immunotherapies of tumor cells with deregulated type I IFN signaling pathways (48, 49) since the 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 TAAs can be used for cross-presentation by APCs, thereby enhancing cancer antigen-directed $CD4^+/CD8^+$ T-cell responses (50). Therefore, the release of CXCR4-A-Fc fusion protein from virally-infected tumor cells could be directly responsible for killing and cross-priming of TAAs in OVV-CXCR4-A-Fc-treated tumors along with inhibition of the immunosuppressive network.

Because innate-resistance of non-responding tumors together with immunosuppressive TME negatively affect immunotherapy treatment, the priming with CXCR4 antagonist-armed virotherapy and peptide-vaccine boost strategy reduces tumor load and drive effective antitumor immunity. Although this strategy provides a foundation and promise as a vaccination strategy, several improvements need to be made to make it applicable to humans. First, the peptide would

need to include best down-selected $CD8^+$ T cell-eliciting epitopes commonly expressed by ovarian cancer and presented by HLA supermotif class I molecules to APC in along with a universal $CD4^+$ helper T lymphocyte epitope and a potent, low toxicity adjuvant (47), since numerous clinical trials conducted with polyI:C (51) showed its systemic cytokine toxicity (52). The phenotype of WT1 $tetramer^+$ cells within the Ly6C population of $CD8^+$ T cells at the tumor sites needs to be investigated because of the association of Ly6C with central memory $CD44^{hi}CD62L^+$, effector $CD44^{hi}CD62L^-$ and to a lesser extent the naïve $CD44^{lo}CD62L^+$ T cell subsets (43). The highest accumulation of the double-positive cells after the combination treatment suggests that infiltration of the antigen-experienced cells in the TME could be driven by the *in situ* vaccination as well as cytokines produced by the expanded $CD103^+$ DCs. This could lead to the generation of T cells subsets with different antitumor efficacy and persistence, which could be evaluated adoptively transferred to congenically distinguished tumor-bearing mice. Furthermore, because the differentiation status of T cells affects antitumor efficacy associated with expression of inhibitory receptor programmed death 1 (PD-1) or the ligand programmed death ligand 1 (PD-L1) (53), further analyses are needed to determine whether addition of anti-PD-L1 antibody treatment should promote the persistence and effector function of tumor-specific $CD8^+$ T cell immune responses. Altogether, the results of our studies argue that strategies that reduce tumor load along with the immunosuppressive network in the TME and restore both the recruitment and activation of $CD103^+$ DCs provide foundation for effective *in situ* vaccination with tumor associated antigens and has the potential to transform clinical responses to immune checkpoint inhibitor treatments.

Materials and Methods

Animals and Cell Lines.

Female C57BL/6 mice were obtained from Charles River Laboratories. B6.129S(C)-*Batf3*^{tm1Kmm}/J mice were purchased from the Jackson Laboratory. Experimental procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the RPCI. The parental ID8 mouse ovarian epithelial cells derived from spontaneous malignant transformation of C57BL/6 MOSE cells (54). Human HuTK$^-$ 143 fibroblasts, human cervical carcinoma HeLa cells, and African green monkey cell line CV-1 were obtained from the American Type Culture Collection.

Viruses.

All vaccinia viruses used in this study are 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 the EGFP, Fc portion of murine IgG2a and the CXCR4-A-Fc fusion protein has been described (9). The CXCR4-A-Fc fusion protein was collected in supernatants of infected HuTKP-P 143 cells and purified on protein G column as described (9).

ELISA.

Concentrations of the soluble CXCR4-A-Fc 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 MEGISIYTSDNYTEEMGSGD YDSMKEPCFREENANFNKIFLPTIYS (Abcam) and Fc portion-specific HRP-conjugated

antibody. The reaction was developed with 1-Step™ Ultra TMB-ELISA reagent (Thermo Scientific). In parallel, protein levels in each sample were determined by Bradford method with Protein Assay Dye Reagent (Bio-Rad).

Generation of BM-derived DC and *In Vitro* Phagocytosis Assays.

BM cells were flushed from the tibias and femurs of C57BL/6 mice and cultured in medium supplemented with 10 ng/ml of GM-CSF for 6 days as described (55). After 7 days, the non-adherent and loosely adherent cells were harvested, washed and co-cultured with cell tracker-blue CMF_2HC (Thermo Fisher Scientific)-labeled tumor cells (1:1 ratio) for 12 h. At the end of the incubation, cells were washed and stained with CD11c-APC antibody for flow cytometry analysis of double positive cells (10).

Treatment of Established Tumors.

C57BL/6 mice (n = 6 - 10) were injected i.p. with 3 x 10^5 ID8-T cells. Treatment with OVV-CXCR4-A-Fc or OVV-Fc (10^8 PFU delivered i.p.) was initiated 10 days later. Tumor progression was monitored by bioluminescence imaging using the Xenogen IVIS Imaging System (PerkinElmer) as described (10). Control mice received PBS or UV-inactivated virus. At the end of the experimental period corresponding to the development of bloody ascites in control mice, the 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, FLT3L (Biolegend) at 5 μg/injection was delivered i.p. for four consecutive days beginning on day 8 after virotherapy treatment, followed by the WT1-specific epitope-based vaccine (50 μg/injection; GenScript) two days after the FLT3L treatment. Eight hours later, 50 μg/mouse of

pI:C (Sigma) was delivered i.p. and progression of tumor growth was analyzed by bioluminescence.

Flow cytometry.

The phenotypic analysis of tumor-infiltrating T cells and myeloid cells was performed on single-cell suspensions prepared from peritoneal fluids collected 8 days after completion of the treatments. All antibodies were purchased from BD Pharmingen, eBioscience, and Biolegend, and a PE-labeled $WT1_{126-134}$/H-$2D^b$ tetramer was obtained from (MHC Tetramer Production Facility, Baylor College of Medicine). For surface staining, cells were incubated with Fc blocker (anti-CD16/CD32 mAb) for 10 min followed by Live/Dead Fixable Violet Dead Cell stain kit (Thermo Fisher Scientific) to assess live/dead cells. Percentages of $CD4^+$ T cells expressing Foxp3 were determined by intracellular staining using BD Pharmingen™ Transcription Factor Buffer Set (BD Biosciences) according to the manufacturer's protocol. Background staining was assessed using isotype control antibodies. Cells were analyzed on a LRS II flow cytometer (BD Biosciences). Data analysis was performed using WinList 3D 7.1 (Verity Software House, Topsham, ME).

Statistical analysis.

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc.,). 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 the survival across

groups were assessed using the log-rank Mantel-Cox method. The threshold for statistical significance was set to $P < 0.05$.

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The authors declare no competing financial interest.

References

1. Hammerich L, Binder A, & Brody JD (2015) In situ vaccination: Cancer immunotherapy both personalized and off-the-shelf. *Mol Oncol* 9(10):1966-1981.
2. Galluzzi L, Buque A, Kepp O, Zitvogel L, & Kroemer G (2017) Immunogenic cell death in cancer and infectious disease. *Nature reviews. Immunology* 17(2):97-111.
3. Guo ZS, *et al.* (2017) Oncolytic Immunotherapy: Conceptual Evolution, Current Strategies, and Future Perspectives. *Frontiers in immunology* 8:555.
4. Lichty BD, Breitbach CJ, Stojdl DF, & Bell JC (2014) Going viral with cancer immunotherapy. *Nature reviews. Cancer* 14(8):559-567.
5. Kaufman HL, Kohlhapp FJ, & Zloza A (2015) Oncolytic viruses: a new class of immunotherapy drugs. *Nat Rev Drug Discov* 14(9):642-662.
6. Kirn DH & Thorne SH (2009) Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nature reviews. Cancer* 9(1):64-71.
7. Andtbacka RH, *et al.* (2015) Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 33(25):2780-2788.
8. Gil M, *et al.* (2014) CXCL12/CXCR4 blockade by oncolytic virotherapy inhibits ovarian cancer growth by decreasing immunosuppression and targeting cancer-initiating cells. *J Immunol* 193(10):5327-5337.
9. Gil M, Seshadri M, Komorowski MP, Abrams SI, & Kozbor D (2013) Targeting CXCL12/CXCR4 signaling with oncolytic virotherapy disrupts tumor vasculature and inhibits breast cancer metastases. *Proceedings of the National Academy of Sciences of the United States of America* 110(14):E1291-1300.

10. Komorowski MP, *et al.* (2016) Reprogramming antitumor immunity against chemoresistant ovarian cancer by a CXCR4 antagonist-armed viral oncotherapy. *Molecular therapy oncolytics* 3:16034.

11. Heo J, *et al.* (2013) Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nature medicine* 19(3):329-336.

12. Zeh HJ, *et al.* (2015) First-in-man study of western reserve strain oncolytic vaccinia virus: safety, systemic spread, and antitumor activity. *Molecular therapy : the journal of the American Society of Gene Therapy* 23(1):202-214.

13. Downs-Canner S, *et al.* (2016) Phase 1 Study of Intravenous Oncolytic Poxvirus (vvDD) in Patients With Advanced Solid Cancers. *Molecular therapy : the journal of the American Society of Gene Therapy* 24(8):1492-1501.

14. Liu Z, Ravindranathan R, Kalinski P, Guo ZS, & Bartlett DL (2017) Rational combination of oncolytic vaccinia virus and PD-L1 blockade works synergistically to enhance therapeutic efficacy. *Nature communications* 8:14754.

15. Takeda Y, *et al.* (2017) A TLR3-Specific Adjuvant Relieves Innate Resistance to PD-L1 Blockade without Cytokine Toxicity in Tumor Vaccine Immunotherapy. *Cell reports* 19(9):1874-1887.

16. Kryczek I, *et al.* (2005) CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. *Cancer research* 65(2):465-472.

17. Kryczek I, Wei S, Keller E, Liu R, & Zou W (2007) Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *Am J Physiol Cell Physiol* 292(3):C987-995.

18. Orimo A, *et al.* (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 121(3):335-348.

19. Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, & Kalinski P (2011) PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res* 71(24):7463-7470.

20. Zou W, *et al.* (2001) Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells. *Nature medicine* 7(12):1339-1346.

21. Righi E, *et al.* (CXCL12/CXCR4 blockade induces multimodal antitumor effects that prolong survival in an immunocompetent mouse model of ovarian cancer. *Cancer research* 71(16):5522-5534.

22. Curiel TJ, *et al.* (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature medicine* 10(9):942-949.

23. Salmon H, *et al.* (2016) Expansion and Activation of CD103(+) Dendritic Cell Progenitors at the Tumor Site Enhances Tumor Responses to Therapeutic PD-L1 and BRAF Inhibition. *Immunity* 44(4):924-938.

24. Merad M, Sathe P, Helft J, Miller J, & Mortha A (2013) The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annual review of immunology* 31:563-604.

25. Guilliams M, *et al.* (2014) Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nature reviews. Immunology* 14(8):571-578.

26. Fuertes MB, *et al.* (2011) Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *The Journal of experimental medicine* 208(10):2005-2016.

27. Corrales L, Matson V, Flood B, Spranger S, & Gajewski TF (2017) Innate immune signaling and regulation in cancer immunotherapy. *Cell research* 27(1):96-108.

28. Spranger S, Dai D, Horton B, & Gajewski TF (2017) Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer cell* 31(5):711-723 e714.

29. Broz ML, *et al.* (2014) Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer cell* 26(5):638-652.

30. Hashimoto D, Miller J, & Merad M (2011) Dendritic cell and macrophage heterogeneity in vivo. *Immunity* 35(3):323-335.

31. van Vloten JP, Workenhe ST, Wootton SK, Mossman KL, & Bridle BW (2018) Critical Interactions between Immunogenic Cancer Cell Death, Oncolytic Viruses, and the Immune System Define the Rational Design of Combination Immunotherapies. *J Immunol* 200(2):450-458.

32. Sistigu A, *et al.* (2014) Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy. *Nature medicine* 20(11):1301-1309.

33. Sukkurwala AQ, *et al.* (2014) Immunogenic calreticulin exposure occurs through a phylogenetically conserved stress pathway involving the chemokine CXCL8. *Cell death and differentiation* 21(1):59-68.

34. Garg AD & Agostinis P (2016) Editorial: Immunogenic Cell Death in Cancer: From Benchside Research to Bedside Reality. *Frontiers in immunology* 7:110.

35. Garg AD, Romano E, Rufo N, & Agostinis P (2016) Immunogenic versus tolerogenic phagocytosis during anticancer therapy: mechanisms and clinical translation. *Cell death and differentiation* 23(6):938-951.

36. Liu K & Nussenzweig MC (2010) Origin and development of dendritic cells. *Immunol Rev* 234(1):45-54.

37. Hylander B, *et al.* (2006) Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. *Gynecologic oncology* 101(1):12-17.

38. Bogunovic D, *et al.* (2011) TLR4 engagement during TLR3-induced proinflammatory signaling in dendritic cells promotes IL-10-mediated suppression of antitumor immunity. *Cancer research* 71(16):5467-5476.

39. Longhi MP, *et al.* (2009) Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *The Journal of experimental medicine* 206(7):1589-1602.

40. Matsumoto M & Seya T (2008) TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev* 60(7):805-812.

41. Mikucki ME, *et al.* (2015) Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nature communications* 6:7458.

42. Salerno EP, Olson WC, McSkimming C, Shea S, & Slingluff CL, Jr. (2014) T cells in the human metastatic melanoma microenvironment express site-specific homing receptors and retention integrins. *International journal of cancer* 134(3):563-574.

43. DeLong JH, *et al.* (2018) Cytokine- and TCR-Mediated Regulation of T Cell Expression of Ly6C and Sca-1. *J Immunol* 200(5):1761-1770.

44. Gupta SK & Pillarisetti K (1999) Cutting edge: CXCR4-Lo: molecular cloning and functional expression of a novel human CXCR4 splice variant. *J Immunol* 163(5):2368-2372.

45. Alexander J, *et al.* (2002) A decaepitope polypeptide primes for multiple CD8+ IFN-gamma and Th lymphocyte responses: evaluation of multiepitope polypeptides as a mode for vaccine delivery. *J Immunol* 168(12):6189-6198.

46. Livingston B, *et al.* (2002) A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. *J Immunol* 168(11):5499-5506.

47. El Bissati K, *et al.* (2016) Adjuvanted multi-epitope vaccines protect HLA-A*11:01 transgenic mice against Toxoplasma gondii. *JCI Insight* 1(15):e85955.

48. Xia T, Konno H, Ahn J, & Barber GN (2016) Deregulation of STING Signaling in Colorectal Carcinoma Constrains DNA Damage Responses and Correlates With Tumorigenesis. *Cell reports* 14(2):282-297.

49. Xia T, Konno H, & Barber GN (2016) Recurrent Loss of STING Signaling in Melanoma Correlates with Susceptibility to Viral Oncolysis. *Cancer research* 76(22):6747-6759.

50. Bournazos S & Ravetch JV (2015) Fcgamma receptor pathways during active and passive immunization. *Immunol Rev* 268(1):88-103.

51. Galluzzi L, *et al.* (2012) Trial Watch: Experimental Toll-like receptor agonists for cancer therapy. *Oncoimmunology* 1(5):699-716.

52. Levine AS, Sivulich M, Wiernik PH, & Levy HB (1979) Initial clinical trials in cancer patients of polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine, in carboxymethylcellulose [poly(ICLC)], a highly effective interferon inducer. *Cancer research* 39(5):1645-1650.

53. Seifert AM, *et al.* (2017) PD-1/PD-L1 Blockade Enhances T-cell Activity and Antitumor Efficacy of Imatinib in Gastrointestinal Stromal Tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research* 23(2):454-465.

54. Janat-Amsbury MM, Yockman JW, Anderson ML, Kieback DG, & Kim SW (2006) Comparison of ID8 MOSE and VEGF-modified ID8 cell lines in an immunocompetent animal model for human ovarian cancer. *Anticancer research* 26(4B):2785-2789.

55. Gil M, *et al.* (2009) Targeting a mimotope vaccine to activating Fcgamma receptors empowers dendritic cells to prime specific CD8+ T cell responses in tumor-bearing mice. *J Immunol* 183(10):6808-6818.

Figure Legends

Fig. 1. Antitumor activity of the CXCR4 antagonist-armed oncolytic virotherapy against orthotopic ID8-T ovarian tumor in syngeneic mice. C57BL/6 female mice (n = 8) were challenged i.p. with ID8-T tumor cells ($3x10^5$ cells). The tumor-bearing mice were treated with sCXCR4-A-Fc (10 μg/injection for 7 days), OVV-EGFP, or OVV-CXCR4-A-Fc (10^8 PFU) injected i.v. (***A***) and i.p. (***B***) 10 days after tumor challenge. Control mice were treated with PBS. Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. $*P < 0.05$, $**P < 0.01$. Accumulation of the CXCR4-A-Fc antagonist in tumor tissues, sera and lymphoid organs after i.v. (***C***) and i.p. (***D)*** delivery of OVV-CXCR4-A-Fc or the s CXCR4-A-Fc fusion protein to ID8-T tumor-bearing mice. Concentrations of CXCR4-A-Fc fusion protein were determined on day 8 after the treatment by ELISA in sera, and cell lysates of tumor, liver, BM, lymph nodes, and spleen tissues after normalization to total protein content. Data are presented as the mean ± SD of five mice per group. $**P < 0.01$, $***P < 0.001$.

Fig. 2. Evaluation of immune infiltrates in ascites-derived tumors or peritoneal washes after treatments with the CXCR4 antagonist delivered as the sCXCR4-A-Fc fusion protein or by OVV-CXCR4-A-Fc oncolytic virotherapy. Additional tumor-bearing mice were treated with OVV-EGFP or PBS. Recruitment of leucocytes ($CD45^+$) (***A***), neutrophils/G-MDSCs ($CD11b^+Ly6G^{hi}Ly6C^{lo}$) (***B***), pDCs ($B220^{hi}Ly6C^{hi}CD11c^{lo}$) (***C***), Tregs ($CD4^+CD25^+Foxp3^+$) (***D***), $CD4^+$ (***E***) and $CD8^+$ (***F***) T cells, TAM1 ($CD45^+CD11b^{hi}Ly6C^-MHCII^+F4/80^+CD11c^{lo}$) (***G***), TAM2 ($CD45^+CD11b^{lo}Ly6C^-MHCII^{hi}F4/80^{hi}CD11c^{hi}$) (***H***), $CD11b^+$DCs ($CD45^+CD11b^{hi}Ly6C^-MHCII^{hi}F4/80^{lo}CD24^{hi}CD103^{lo}$) (***I***), $CD103^+$DCs ($CD45^+CD11b^{lo}LY6C^-MHCII^+F4/80^{lo}CD24^{hi}CD103^{hi}$) (***J***) were analyzed by flow cytometry 8 days after treatments, as described in the Materials and Methods. Data are mean ± SD of three or four independent experiments. $**P < 0.01$, $***P < 0.001$.

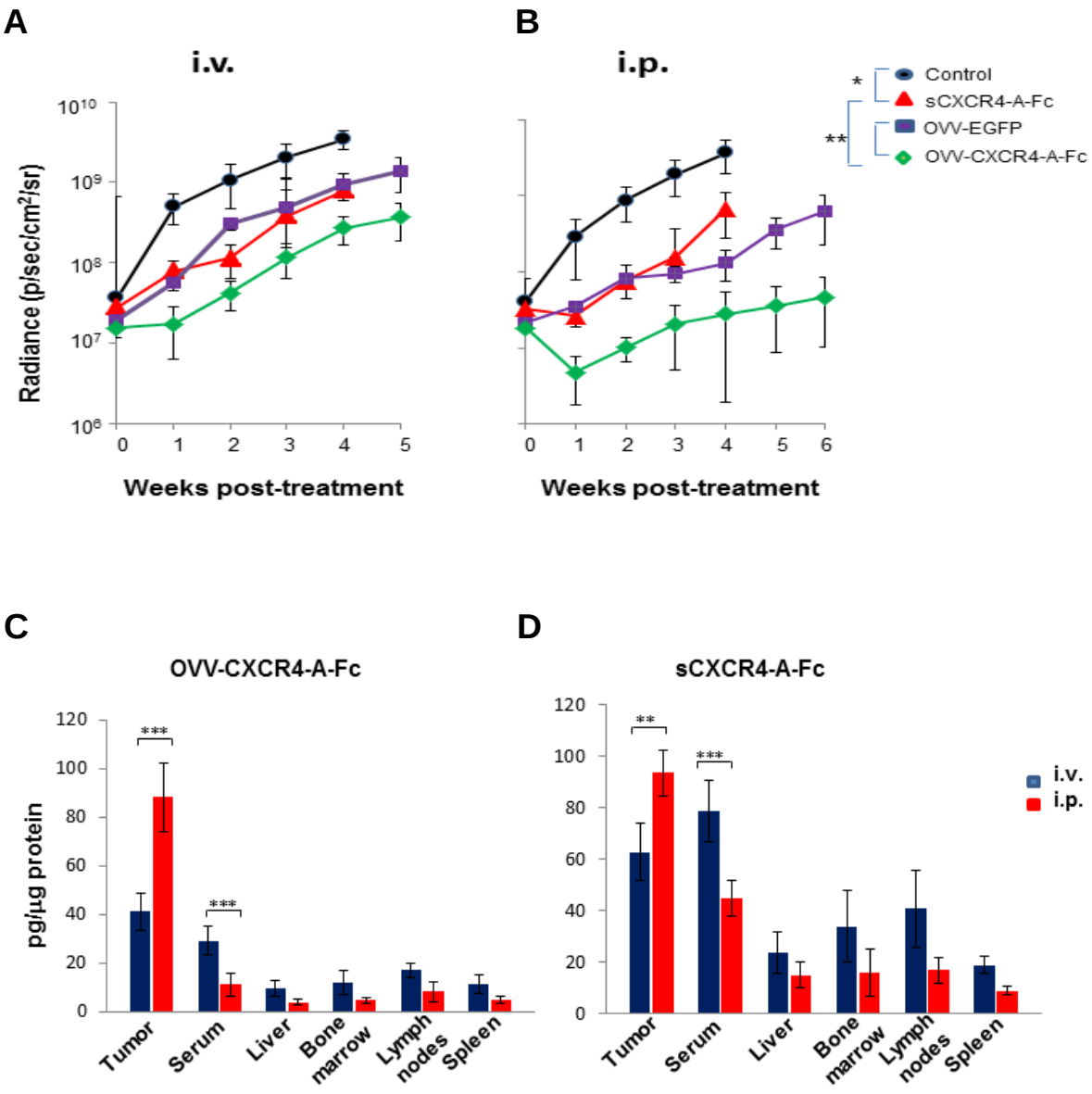
Fig. 3. The CXCR4 antagonist induces apoptosis associated with phagocytosis of tumor cell debris by BM-derived $CD11c^+$ DCs. (***A***) Cell death of ID8-R tumor cells treated with the sCXCR4-A-Fc fusion protein (10 μg/ml), OVV-Fc or OVV-CXCR4-A-Fc (MOI = 1) was determined by staining with Annexin V-FITC 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 CMF2HC-labeled ID8-T tumor cells treated with the sCXCR4-A-Fc fusion protein, OVV-Fc, or OVV-CXCR4-A-Fc by $CD11c^+$ DCs was measured after 12 h of incubation by flow cytometry. All tumor cell cultures were treated with UV and psolaren to eliminate the virus before combining with DCs. Tumor cells receiving UV and psolaren treatment were included as additional controls. The percentages of CD11c-expressing DCs taking up tumor cells are indicated. One representative experiment of three independent experiments performed is shown.

Fig. 4. Expansion of $CD103^+$ DCs at the tumor site by the growth factor FLT3L delays tumor growth and increases phagocytosis of tumor cell debris. (***A***) FLT3L was delivered i.p. to virotherapy-treated ID8-T-bearing mice at 5 μg/mouse for 4 consecutive days, beginning on day 8 after virotherapy treatment. The expansion of $CD11b^+$ DCs, $CD103^+$ DCs, TAM1, TAM2, and monocytes in peritoneal washes were analyzed two days after the treatment by flow cytometry. (***B***) Tumor progression was monitored by bioluminescence. Data points represent mean ± SD. (***C***) $CD45^+$ leukocytes isolated from peritoneal cavities of tumor-bearing mice two days after treatments with OVV-EGFP or OVV-CXCR4-A-Fc alone or in combination with FLT3L were cultured with virally-treated and cell tracker-labeled ID8-T cancer cells. After overnight incubation, the capture of tumor-associated fluorescent debris by $CD103^+$ DCs was analyzed by

multicolor flow cytometry. One representative experiment of three independent experiments performed is shown. (***D***) Percentages of phagocytosis of virally-treated tumor cell debris by $CD103^+$ DCs are presented as mean ± SD.

Fig. 5. Expansion of $CD103^+$ DCs at the tumor site enhances tumor responses to therapeutic WT1-specific peptide vaccine. (***A***) Graphical time line of the treatment scheme in ID8-T tumor-bearing mice. C57BL/6 mice (n = 6 - 10) were injected i.p. with 3 x 10^5 ID8-T cells. Treatment with OVV-CXCR4-A-Fc or OVV-Fc (10^8 PFU delivered i.p.) was initiated 10 days later. To expand $CD103^+$ DCs, FLT3L 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) two days after the last FLT3L injection. To promote the maturation of the mobilized $CD103^+$ DCs, the tumor-bearing mice were treated with pI:C (50 μg/injection) 8 h after WT1 peptide immunization. (***B,C***) Survival was defined as the point at which mice were killed because of extensive tumor burden. Kaplan-Meier survival plots were prepared and significance was determined using the log-rank method. $^{**}P < 0.01$, $^{***}P < 0.001$.

Fig. 6. Evaluation of $CD8^+Ly6C^+$ T cells (*left panel*) and WT1 $tetramer^+$ subset of $CD8^+Ly6C^+$ T cells populations (*right panel*) in ascites-derived tumors or peritoneal washes after combination treatments with: PBS (***A***,***G***); OVV (***B***,***H***); OVV + FLT3L (***C***,***I***); OVV + WT1 (***D***, ***J***); and OVV+ FLT3L + WT1 (***E***, ***K***). Immunofluorescence staining on single-cell suspensions of $CD45^+CD11b^-$ cells with CD8- and Ly6C-specific antibodies was performed 7 days after completion of the treatment depicted in **Fig. 5*A***. One representative experiment of four mice per group performed is shown.


A
i.v.
Radiance (p/sec/cm²/sr)
Weeks post-treatment
B
i.p.
Weeks post-treatment
Control
sCXCR4-A-Fc
OVV-EGFP
OVV-CXCR4-A-Fc
*
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C
OVV-CXCR4-A-Fc
pg/µg protein
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Tumor
Serum
Liver
Bone marrow
Lymph nodes
Spleen
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sCXCR4-A-Fc
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i.v.
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Figure 1

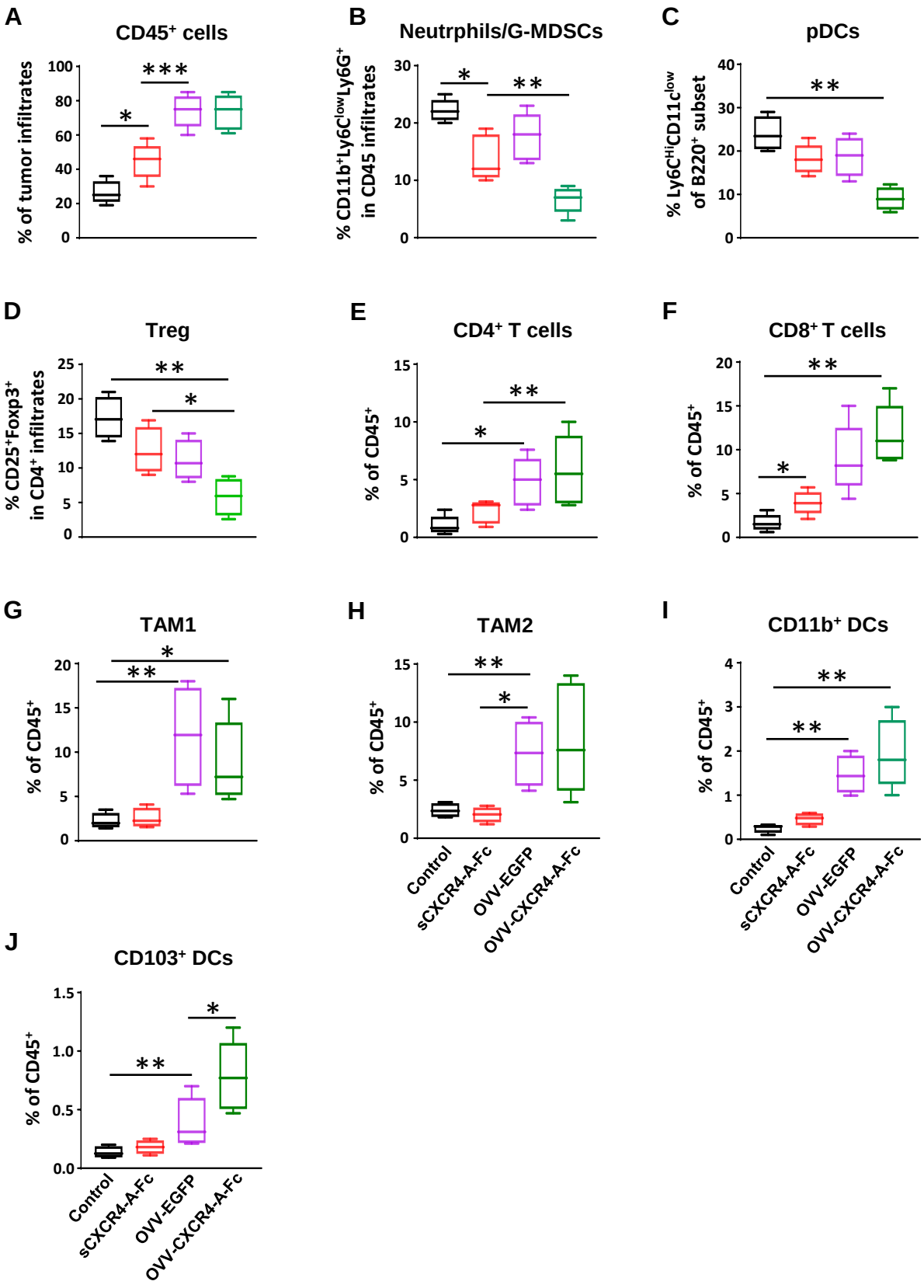

A
CD45+ cells
% of tumor infiltrates
B
Neutrphils/G-MDSCs
% CD11b+Ly6Clow Ly6G+ in CD45 infiltrates
C
pDCs
% Ly6CHiCD11clow of B220+ subset
D
Treg
% CD25+Foxp3+ in CD4+ infiltrates
E
CD4+ T cells
% of CD45+
F
CD8+ T cells
% of CD45+
G
TAM1
% of CD45+
H
TAM2
% of CD45+
I
CD11b+ DCs
% of CD45+
J
CD103+ DCs
% of CD45+
Control
sCXCR4-A-Fc
OVV-EGFP
OVV-CXCR4-A-Fc

Figure 2

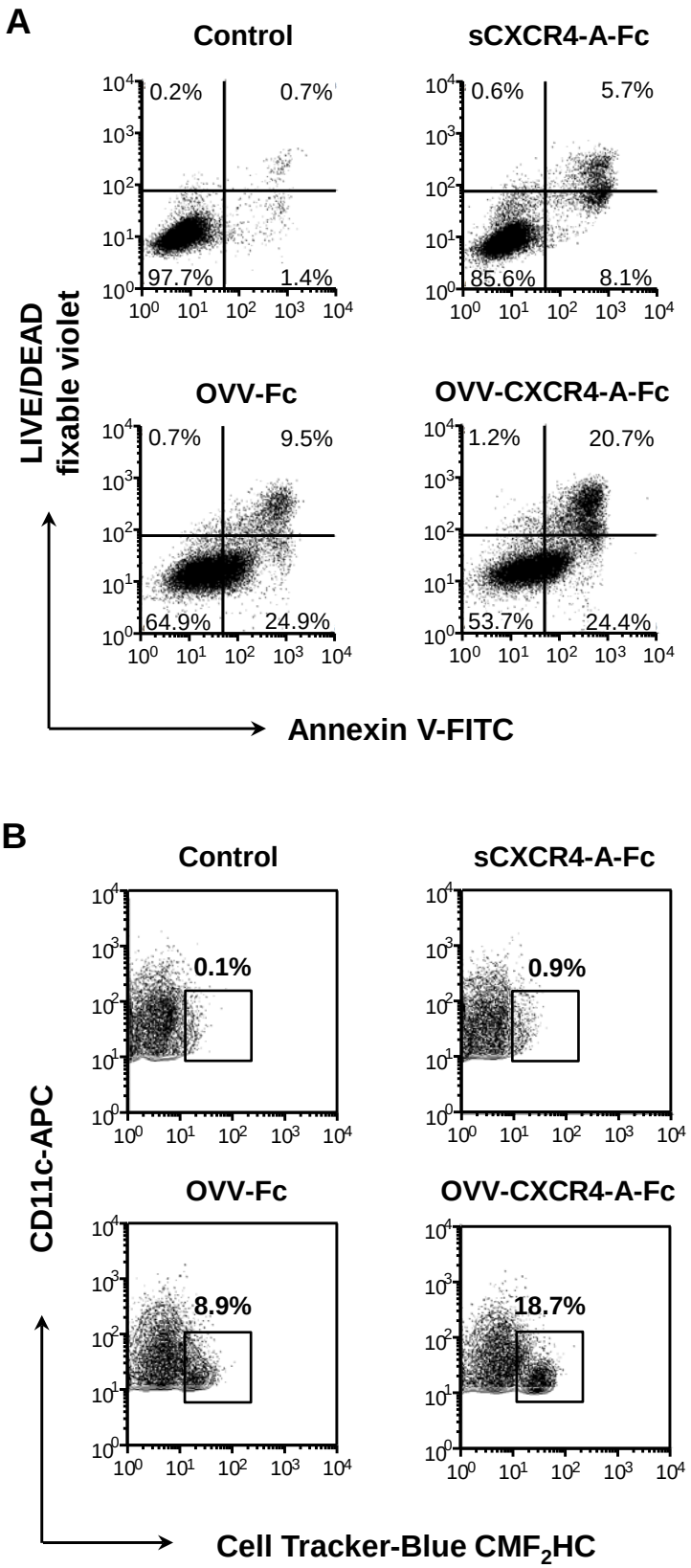

A
Control
sCXCR4-A-Fc
OVV-Fc
OVV-CXCR4-A-Fc
0.2%
0.7%
97.7%
1.4%
0.6%
5.7%
85.6%
8.1%
0.7%
9.5%
64.9%
24.9%
1.2%
20.7%
53.7%
24.4%
LIVE/DEAD fixable violet
Annexin V-FITC
B
Control
sCXCR4-A-Fc
OVV-Fc
OVV-CXCR4-A-Fc
0.1%
0.9%
8.9%
18.7%
CD11c-APC
Cell Tracker-Blue CMF2HC

Figure 3

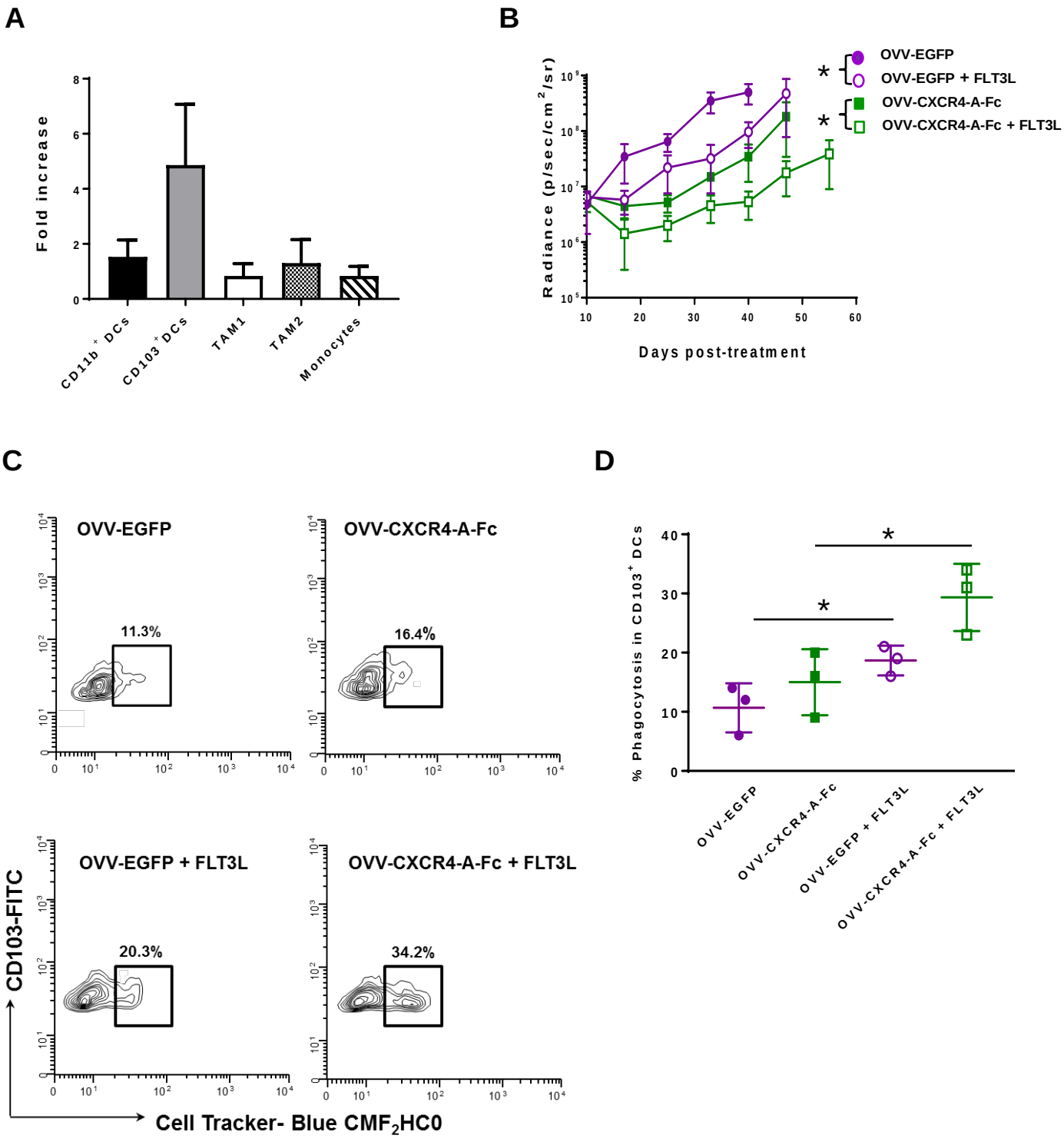

A
Fold increase
CD11b+ DCs
CD103+ DCs
TAM1
TAM2
Monocytes
B
Radiance (p/sec/cm2/sr)
Days post-treatment
OVV-EGFP
OVV-EGFP + FLT3L
OVV-CXCR4-A-Fc
OVV-CXCR4-A-Fc + FLT3L
C
OVV-EGFP
11.3%
OVV-CXCR4-A-Fc
16.4%
OVV-EGFP + FLT3L
20.3%
OVV-CXCR4-A-Fc + FLT3L
34.2%
CD103-FITC
Cell Tracker- Blue CMF2HC0
D
% Phagocytosis in CD103+ DCs
OVV-EGFP
OVV-CXCR4-A-Fc
OVV-EGFP + FLT3L
OVV-CXCR4-A-Fc + FLT3L

Figure 4

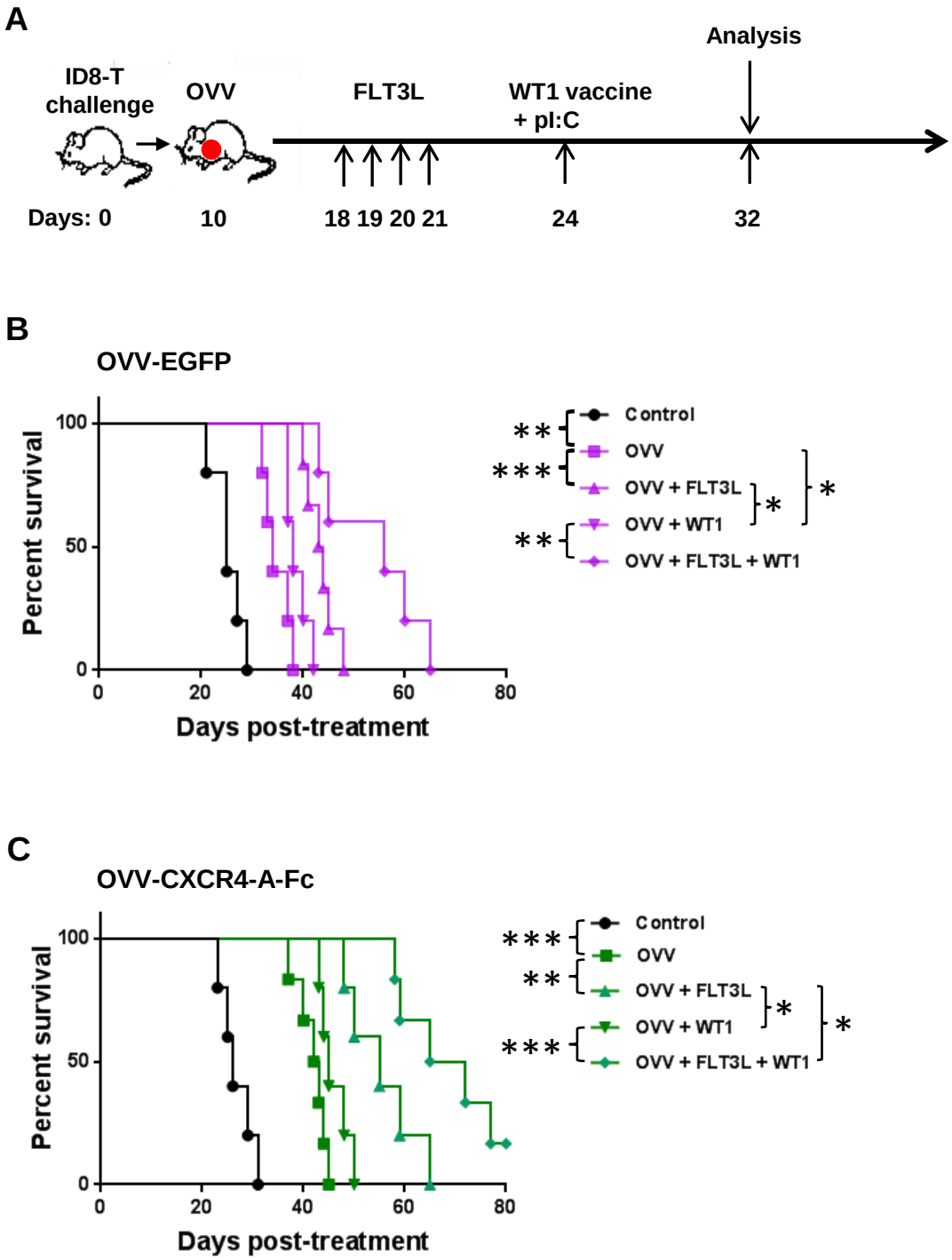
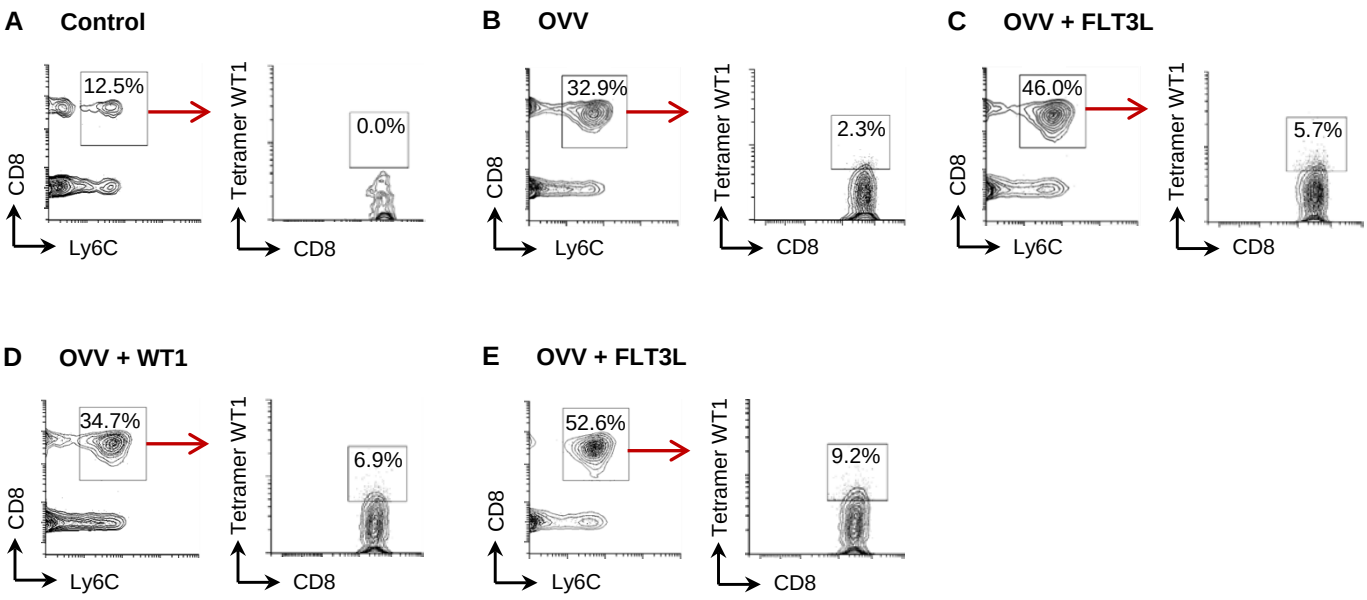

A
ID8-T challenge
OVV
FLT3L
WT1 vaccine + pI:C
Analysis
Days: 0
10
18 19 20 21
24
32
B
OVV-EGFP
Percent survival
Days post-treatment
Control
OVV
OVV + FLT3L
OVV + WT1
OVV + FLT3L + WT1
C
OVV-CXCR4-A-Fc
Percent survival
Days post-treatment
Control
OVV
OVV + FLT3L
OVV + WT1
OVV + FLT3L + WT1

Figure 5

OVV-EGFP



OVV-CXCR4-A-Fc

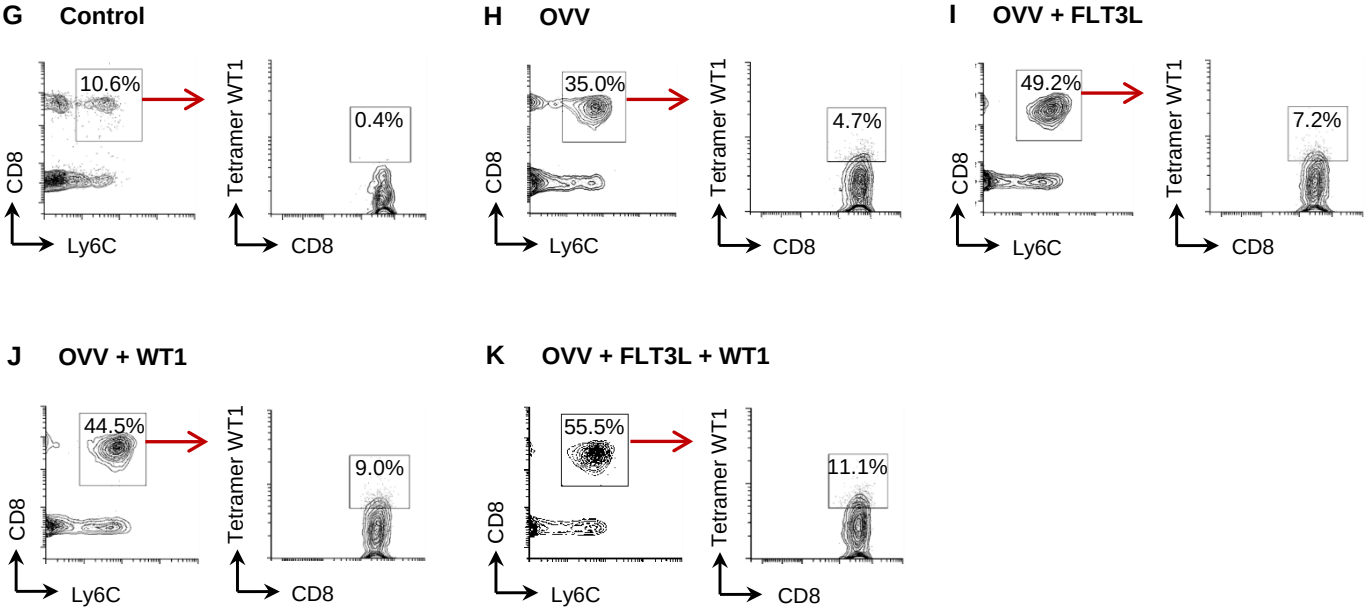


Figure 6