HHS Public Access

Author manuscript

Cancer Gene Ther. Author manuscript; available in PMC 2022 December 02.

Published in final edited form as:

Cancer Gene Ther. 2021 February ; 28(1-2): 98–111. doi:10.1038/s41417-020-0189-4.

Oncolytic Virus Promotes Tumor-Reactive Infiltrating Lymphocytes for Adoptive Cell Therapy

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Abstract

Adoptive cell therapy (ACT) using tumor-specific tumor-infiltrating lymphocytes (TILs) has demonstrated success in patients where tumor-antigen specific TILs can be harvested from the tumor, expanded, and re-infused in combination with a preparatory regimen and IL-2. One major issue for non-immunogenic tumors has been that the isolated TILs lack tumor specificity and thus possess limited *in vivo* therapeutic function. An oncolytic virus (OV) mediates an immunogenic cell death for cancer cells, leading to elicitation and dramatic enhancement of tumor-specific TILs. We hypothesized that the tumor-specific TILs elicited and promoted by an OV would be a great source for ACT for solid cancer. In this study, we show that a local injection of oncolytic poxvirus in MC38 tumor with low immunogenicity in C57BL/6 mice, led to elicitation and accumulation of tumor-specific TILs in the tumor tissue. Our analyses indicated that IL-2-armed OV-elicited TILs contain lower quantities of exhausted PD-1^{hi}Tim-3^{+} $CD8^{+}$ T cells and regulatory T cells. The isolated TILs from IL-2-expressing OV-treated tumor tissue retained high tumor-specificity after expansion *ex vivo*. These TILs resulted in significant tumor regression and improved survival after adoptive transfer in mice with established MC38 tumor. Our study showcases the feasibility of using an OV to induce tumor-reactive TILs that can be expanded for ACT.

Keywords

oncolytic virus; vaccinia virus; IL-2; antitumor adaptive immunity; tumor-specific T cells; expansion ex vivo; adoptive T cell therapy

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Conflicts of Interests: MF, ZL, ZSG and BLB filed a patent partly based on this work. Other authors declare that they have no conflict of interest.

INTRODUCTION

Immunotherapy for cancer has become a reality in the last few years, with the approval by the FDA of a therapeutic cancer vaccine for advanced prostate cancer (Provenge), an oncolytic virus T-VEC for advanced melanoma, two types of CAR T cells for leukemia, and multiple antibody-based drugs of immune checkpoint blockade for a variety of cancers. Immunotherapy is rapidly evolving and treating cancer by activating the patient's immune system presents an attractive therapeutic strategy [1]. One promising strategy is adoptive cell therapy (ACT) using the patient's own TILs – a form of personalized cancer immunotherapy. This approach consists of excising a tumor, then expanding the lymphocytes from the tumor *ex vivo*. The patient is then treated with a non-myeloablative preparative chemotherapy regimen followed by TIL infusion and systemic IL-2 [2]. This approach has been successful in patients with cutaneous melanoma where the tumor mutational load (TMB) is high and tumor reactive lymphocytes are abundant in the tumor. Objective response rates (ORR) were observed ranging from 49% to 72%, with long term durable and potentially curative complete response rates of up to 25% [3], ORR was 42% with an attenuated IL-2 dose in another study [4]. However, most human solid tumors are non-immunogenic and tumor-reactive TIL cannot be isolated or expanded for ACT. Recently, however, ACT of autologous TILs, isolated and separated for tumor-reactive TILs (which made up a small percentage of total TILs), was shown to induce objective tumor regression in patients with metastatic uveal melanoma, a low mutational burden, non-immunogenic tumor [5]. In this trial, there was a strong association between the anti-tumor reactivity of the infused TILs and objective clinical response, suggesting that mechanisms to improve recovery of tumor reactive TILs could lead to more effective therapy for other solid tumors.

There are at least two major hurdles in successful ACT for cancers other than melanoma. First, the source of TILs is a major issue as the majority of human solid cancers are considered poorly immunogenic and very few TILs exist in the tumor tissues [6]. Even if it is possible to isolate TILs in non-immunogenic cancers such as uveal melanoma, most TILs isolated were not tumor-reactive [5]. Second, other than TILs from melanoma patients, there have not been many successful studies of isolation and expansion of tumor-specific TILs. The standard operating procedures for isolation and expansion of tumor-specific TILs *ex vivo* from other types of solid cancers have not been established. This was the case even for relatively high immunogenic renal cell carcinomas until recently [7].

Local immunotherapy, including oncolytic virotherapy, may modulate the tumor immune contexture and convert tumors from immunologically "cold" to "hot" [8–10]. We hypothesized that cytokine armed oncolytic VV could create a local pro-inflammatory environment in otherwise poorly immunogenic tumors leading to tumor reactive T-cells that could be harvested for ACT of cancer. Oncolytic viruses (OVs) selectively infect and/or replicate in cancer cells *in vivo* leaving normal cells unharmed. The immunogenic cell death induced by VV exposes a natural repertoire of tumor-associated antigens in conjunction with danger signals: damage associated molecular pattern molecules (DAMPs) and virus-derived pathogen-associated molecular pattern molecules (PAMPs), and inflammatory cytokines to reverse the tumor induced immunosuppression and elicit anti-tumor cellular immunity [11,

12]. OVs have been shown in both pre-clinical studies [13–16], and clinical trials with T-VEC and Pexa-Vec [9, 17, 18] to induce potent adaptive antitumor immunity contributing to the overall efficacy of the therapy. To further improve the antitumor immunity, investigators have engineered various oncolytic VV to express tumor antigens, T-cell co-stimulatory molecules and inflammatory chemokines and cytokines, and they have been studied for their efficacy and safety in preclinical tumor models [19–22].

In the current study, we utilized relatively low immunogenic MC38 murine colon tumor, which displays low levels of inflammatory infiltrate and tumor-reactive TILs, as a model for study. We present for the first time that oncolytic VVs can induce local tumor-specific TILs, and these TILs can be harvested, expanded *ex vivo*, and utilized for ACT to achieve a therapeutic response in a tumor model with low-immunogenicity. We envision that this approach using OV to induce tumor reactive TILs in low- or non-immunogenic tumors could be translated to cancer patients, and such *ex vivo* expanded TILs could be used for ACT for solid tumors with the potential for long term cure.

MATERIALS AND METHODS

Mice and cell lines.

Female C57BL/6J (B6) mice, age 5–6 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions in the University of Pittsburgh Animal Facility. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Murine colon cancer cell line B16 was originally obtained from American Type Culture Collection (Manassas, VA). Mouse colon cancer cell line MC38-luc was described previously [23]. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1 penicillin/streptomycin (Invitrogen, Carlsbad, CA) in 37°C, 5% CO_2 incubator. All cell lines were tested for mycoplasma once every three months or so, to ensure they were free of the contamination.

Viruses.

WR strain-derived recombinant oncolytic VVs, vvDD, vvDD-CCL5 and vvDD-CXC11, [24–26], vvDD-IL15-Rα [27] and vvDD-IL2 expressing a membrane-bound IL-2 [28], have been constructed and studied. These VVs were amplified in HeLa cells and then purified as previously described [23, 26]. Viral titers were determined by plaque assays in CV-1 cells. For intratumoral injection of the viruses, a dose of 1.0e8 pfu per mouse was used unless indicated otherwise.

Rodent Tumor models and virus treatments.

For subcutaneous (s. c.) tumor model, B6 mice were subcutaneously inoculated with 5.0×10^5 MC38 colon cancer cells. When the tumors reached the size ~ 5 × 5 mm, vvDD, vvDD-IL2, vvDD-CCL5, vvDD-IL15Rα, vvDD-CXC11, or PBS was intratumorally injected at 1.0e8 pfu/tumor. In some experiments human IL-2 has been injected intratumorally at 1.0×10^6 IU per tumor (Prometheus, San Diego, CA). The primary tumor size was measured using an electric caliper in two perpendicular diameters followed by measurement every

other day. Ten days post virus treatment, tumor tissues or spleens were harvest and then single cell suspension was made for T cell separation and further analysis. For peritoneal (i.p.) tumor models, mice were intraperitoneally inoculated with 5.0×10^5 MC38-luc cancer cells and randomized according to tumor size based on live animal IVIS imaging 7 days post tumor cell injection using a Xenogen IVIS 200 Optical In Vivo Imaging System (Caliber Life Sciences, Hoptikon, MA).

Generation of tumor-reactive TILs for adoptive cell transfer.

B6 mice were s.c. inoculated with 5.0×10^5 MC38 cancer cells at the right hind frank. When the tumor area reached 5 × 5 mm, vvDD-IL2 ($1.0e^8$ pfu per tumor) was intratumorally injected. 10 days later tumors were collected, cut into pieces and incubated at 37°C in digestion buffer (Miltenyi Biotec, San Diego, CA) before being mashed over a 100 μM tissue strainer. Lysis of red blood cells was performed using ACK Lysing buffer (Thermo Fisher Scientific, Waltham, MA). For Leukocyte separation, Percoll gradient centrifuge procedure adapted from the protocol by Liu et al. [29] was used. Leucocytes were collected at the interface between 40% and 80% discontinuous Percoll gradient followed by magnetic separation (CD90.2 beads, Miltenyi Biotec). T cells have been cultured in 24-well plates in a concentration with 1.0×10^6 cells per well in RPMI complete media with 30 IU/ml IL-2 (MiltenyiBiotec) and 5 ng/ml IL-7 (BioLegend, San Diego, CA) over 4 days. As control T cells, spleens from non-tumor bearing untreated mouse have been harvested and proceeded to single cell suspension followed by magnetic separation (CD90.2 beads). The control T cells have been cultured under the same conditions as the virus induced T cells. Prior to adoptive cell transfer, T cells have been analyzed for tumor specificity in a co-culture assay. T cells (2.0×10^4 per well, 96-well plate) were either left unstimulated (medium) or challenged with γ-irradiated MC38 tumor cells (2.0×10^4 per well) or irrelevant target cells as γ-irradiated B16 tumor cells (2.0×10^4 per well) or naïve splenocytes (2.0×10^4 per well) from non-tumor-bearing B6 mice in duplicate for 24 h. The plate setup has been used for IFN-γ ELISPOT assay or flow cytometry analysis as described below.

Tumor model, adoptive cell transfer and cytokine administration.

B6 mice were intraperitoneally inoculated with 5.0×10^5 MC38-luc cancer cells and divided into required groups according to tumor growth condition based on live animal IVIS imaging 7 days post tumor cell injection. Grouped mice received 5.0×10^6 TILs that were isolated from vvDD-IL2-treated tumors and then expanded *ex vivo*, naïve T cells from control mice or just PBS. All treated mice also received 5 Gy of sublethal irradiation according to clinical protocols prior to cell transfer and exogenous cytokine support of IL-2 (100,000 IU/mouse, i.p.) for 3 days post transfer every 12 h (Prometheus).

Flow cytometry.

Collected tumor tissues were minced and incubated in RPMI 1640 medium containing 2% FBS, 1mg/mL collagenase IV (Sigma: #C5138), 0.1mg hyaluronidase (Sigma: #H6254), and 200U DNase I (Sigma: #D5025) at 37°C for 1 hours to make single cells. In vitro virus-infected cells or single cells from tumor tissues were blocked with α-CD16/32 Ab (clone 93, eBioscience: #14-0161-85; 1:1000) and then cells were stained with 100 μL Zombie Aqua Fixable Viability Kit cell dye (BioLegend, San Diego, CA, USA) at a dilution

of 1:1000 and left in room temperature for 15 min in the dark. Cells were stained in 100 μL total stain volume (50 μL BV stain buffer, 50 μl 2% FBS) with antibody at a dilution of 1:200 for 30 min on ice in the dark. The sources of antibodies are listed in Supplementary Table 1. The intracellular staining kit for Foxp3 and IFN-γ was purchased from BioLegend. Cells were then washed once with FACS buffer and fixed in 1% paraformaldehyde (EK Industries, Joliet, IL, USA) before being stored at 4°C overnight and acquired the next day on a BD LSR Fortessa II analyzer (BD Biosciences, San Jose, CA, USA). Data were analyzed using flowJo cytometer software.

The enzyme-linked immunospot (ELISpot) assay.

Collected tumor tissues were cut into pieces and incubated at 37°C in digestion buffer (Miltenyi Biotec, San Diego, CA) before being mashed over a 100 μM tissue strainer. Lysis of red blood cell was performed using ACK Lysing buffer (Thermo Fisher Scientific, Waltham, MA) and single cell suspension was proceeded by straining cell suspension over 40 μM filter. Isolation of $CD8^+$ TILs was performed using negative α-mouse CD8 microbeads isolation protocol (Miltenyi Biotec, San Diego, CA). Ninety-six well plates (MAHAS4510, Millipore, Burlington, MA) were coated with anti-mouse IFN-γ mAb 15mg/ml (clone AN18, Mabtech Inc., Cincinnati, OH). T cells (2.0×10^4 per well) were either left unstimulated (medium) or challenged with γ-irradiated MC38 tumor cells (2.0×10^4 per well in 96-well plate) or irrelevant target cells as γ-irradiated B16 tumor cells (2.0×10^4 per well) or naïve splenocytes (2.0×10^4 per well) from non-tumor-bearing B6 mouse in duplicate for 24 h. After appropriate washes, biotylated secondary antibody (clone R4–6A2-biotin, Mabtech, Inc) was added and incubated at room temperature for 2 h. Spot development followed using Vectastain Elite ABC and AEC peroxidase substrate kit (Vector Laboratories, Inc. Burlingame, CA). Number of IFN-γ spots were analyzed by ImmunoSpot™ (Cellular Technology, Ltd., Shaker Heights, OH).

To determine MC38-reactive responses the average value of spots from control wells were substracted from the number of MC38 challenged wells.

Immunofluorescence staining.

Resected tumors were fixed for 2 h in 2% Paraformaldehyde and incubated in 30% sucrose overnight. Sections were cut (5 μm) and stained with combined primary antibodies CD3 Alexa 488 (100212, BioLegend), CD4 Alexa 594 (100446, BioLegend) and CD8 Alexa 647 (100727, BioLegend) and nuclei were labeled with Hoechst dye (bis benzimide, Sigma B-2283–1 mg/100 ml in dH20). Images were acquired digitally from 9 fields under each condition. Density of positive cells was evaluated by automated image analysis using Nikon Elements (Nikon Instruments Inc, Melville, NY). Percentage of $CD3^+$ T cells, $CD3^+CD4^+$, $CD3^+CD8^+$ T cells per area has been calculated by number of cells positive for the antibody versus the total number of cells.

Long-term survival of mice.

The health and survival of treated mice was closely monitored. All mice bearing subcutaneous or peritoneal tumors were monitored via caliper measurements for changes in tumor size or abdominal girth. Mice were dead naturally due to the disease or sacrificed

when their subcutaneous tumor size exceeded 20 mm in diameter or when abdominal girth exceeded 1.5× the original measurement.

Statistics.

For the majority of experiments, the numbers of mice per group was equal to, or bigger than 5. We have chosen these sample sizes to ensure adequate statistical power either by pilot studies or previous studies we had conducted. For tumor-bearing mice, right before the treatment, they were pooled and then randomly distributed into different groups. For most of the animal experiments, it is not blinded. In rare occasions, if the value of one individual is considered to be an outlier (the value of 2 times of s.d outside of the mean), we might have excluded the value from the presented data.

Statistical analyses were performed using unpaired Student's *t* test for two group comparison. For multiple group comparison One-way ANOVA were used where *p* value is adjusted for multiple test by Dunnett method (GraphPad Prism version 5). Animal survival is presented using Kaplan-Meier survival curves and compared by using log rank test (GraphPad Prism version 5). Value of $p < 0.05$ is considered to be statistically significant, and all *p* values were two sided. In the figures, the standard symbols were used: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; and NS: not significant.

RESULTS

Oncolytic VVs elicit and attract high numbers of tumor-reactive TILs

We engineered several vvDD-based oncolytic VVs expressing different cytokines/chemokines (vvDD-CCL5, vvDD-CXC11, vvDD-IL15Rα, vvDD-IL2) to enhance the anti-tumor immune response and to attract T cells into the tumor tissue. We first explored a variety of time points after injection and found that 10 days was the optimal time to harvest tumor reactive T-cells from the tumor microenvironment (data not shown). We studied TILs in implanted subcutaneous MC38 tumors 10 days after intratumoral treatment with either PBS, vvDD, vvDD-CCL5, vvDD-CXC11, vvDD-IL15Rα or vvDD-IL2 (Fig. 1A). $CD8^+$ TILs from these tumors were isolated and analyzed using an IFN-γ ELISpot assay against γ-irradiated MC38 tumor cells. From control tumors injected with PBS, there were no tumor-reactive T cells found in the tumor. In contrast, each of the virus-treated tumors demonstrated a significant increase in tumor-reactive $CD8^+$ TILs over control ($p < 0.05$). vvDD-IL2-treated tumors had high frequencies of tumor-reactive $CD8^+$ TILs compared to control (Fig. 1A). Also, vvDD-IL2 yielded one of the most promising therapeutic results in a separate study with MC38 colon tumor models [28]. Thus, we have picked vvDD-IL2 for the rest of our study here.

To investigate whether the OV-induced $CD8^+$ TILs were specific for the injected tumor, the experiment was repeated with the murine tumor B16 and naïve splenocytes as additional control targets. Subcutaneous MC38 tumors were injected with vvDD-IL2 or PBS, and 10 days later the TILs were harvested. $CD8^+$ T cells from treated tumors were again analyzed using an IFN-γ ELISPOT assay against γ-irradiated MC38 tumor cells, B16 tumor cells, or naïve splenocytes from a non-tumor-bearing B6 mouse (Fig. 1B). vvDD-IL2-treated

tumors again demonstrated a significant increase in MC38 tumor reactive $CD8^+$ T cells. The vvDD-IL2-induced $CD8^+$ TILs were highly reactive and specific against MC38 tumor cells compared to the irrelevant target cells. In summary, vvDD-IL2 injection into MC38 tumors induced high levels of MC38 specific TILs.

We repeated the experiment again with vvDD and IL-2 protein injection as additional controls, using an effective dose based on a prior study [30], and examined both systemic and intratumoral T-cells for tumor reactivity. MC38 s.c. tumor-bearing mice were treated intratumorally with PBS, IL-2, vvDD or vvDD-IL2. Ten days later, both splenocytes and the $CD8^+$ fraction of TILs were isolated and analyzed by IFN-γ ELISPOT assays. Both viruses induced greater frequency of tumor reactive T cells in the spleen compared to controls as shown by IFN-γ release (Fig. 1C). Further, each of the viruses induced a greater frequency of tumor-reactive TILs compared with PBS, and the vvDD-IL-2 was superior to vvDD or IL-2 alone in promoting recovery of antitumor $CD8^+$ lymphocytes from the tumor (Fig. 1D).

While vvDD-IL2 in the above studies induced a higher percentage of tumor-reactive $CD8^+$ TILs amongst recovered $CD8^+$ cells, we also hypothesized that the absolute numbers of $CD8^+$ T cells were increased after viral infection. We determined the quantity of $CD4^+$ and $CD8^+$ T cells in the tumor at day 10 after treatment, using two methods of analyses. First, tumors were analyzed by immunofluorescence staining for total $CD3^+$ infiltrating cells, $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells (Fig. 2). vvDD-IL2 promoted significant increases of total $CD3^+$ and $CD3^+CD8^+$ cell infiltration in the tumor tissue compared to PBS treatment. Oncolytic vvDD-IL2 treatment presented a trend towards increased $CD3^+CD4^+$ TILs (% $CD3^+CD4^+$ T cells: vvDD-IL2 vs PBS: $p = 0.0588$). Second, T cells were purified from tumor tissues and quantified as per gram of tumor tissue, and similar patterns were observed (Suppl. Fig. 1). Cumulatively, these results indicate that viral treatment, especially vvDD-IL2, elicits potent adaptive antitumor immunity leading to an increased number of $CD3^+CD8^+$ T cells in the tumor tissues, as well as an increased percentage of tumor-reactive $CD3^+CD8^+$ T cells in the tumor.

OV-induced TILs profiling indicated a strong pro-antitumor immunity.

We then analyzed the status of T cells in the tumor tissues by flow cytometry (Fig. 3). To start with, we looked at the splenocytes and set them as control for comparison (Fig. 3A). In the spleen, $CTLA\text{-}4^+CD8^+$ T cells were slightly reduced in both groups treated with OVs. $Tim\text{-}3^+CD8^+$ T cells were not changed, while $PD\text{-}1^{hi}Tim\text{-}3^+$ $CD8^+$ T cells were slightly reduced. The $PD\text{-}1^{hi}Tim\text{-}3^+$ $CD8^+$ T cells represent the highly exhausted cell population [31]. Interestingly, T*reg* ($Foxp3^+CD4^+$ T cells) cells were reduced in the mice treated with vvDD-IL2, but not in the group treated with vvDD. Then we analyzed the same set of markers in TILs (Fig. 3B). We observed increased $CTLA\text{-}4^+$ or $TIM\text{-}3^+$ $CD8^+$ T cells in the virus-treated groups. Surprisingly, when we examined $PD\text{-}1^{hi}Tim\text{-}3^+$ $CD8^+$ T cells, considered to be highly exhausted cells, we saw a very exciting pattern of changes. The group treated with vvDD exhibited a reduction of this exhausted cells, and this reduction was much greater in the mice treated with vvDD-IL2 ($p < 0.01$). When we analyzed T*reg* cells, the same patterns of reduction were observed.

During T cell activation, there is a dynamic transition from Tn to Tcm to Tem, and finally effector T cells. We therefore examined the $CD8^+$ T cell subsets (Fig. 3C). First, there was a large percentage of Tn (naïve T cells) in the PBS group, while Tn was greatly reduced in the two groups treated with OVs. In the Tcm subset, there was an increase in the groups treated with OVs, and finally, there was a large increase of the Tem, from ~17% in PBS group to ~43% in the groups treated with OVs. When we analyzed the populations of $4–1BB^+CD8^+$ T cells, there were clearly an enhancement in the OV-treated groups ($p < 0.001$, compared to PBS group). These results showed convincingly that OV activated and promoted differentiation of CD8+ T cells toward effector T cells.

We have also conducted preliminary studies on the specificity of TILs recognizing certain antigens. We pulsed naïve splenocytes with $H\text{-}2K^b$-restricted epitope peptides, $p15E_{604–611}$ (retrovirally-encoded tumor antigen) [32], $B8R_{20–27}$ (dominant vaccinia virus antigen) [33], and β-galactosidase-derived epitope (DAPIYTNV), then incubated with TILs isolated from MC38-tumor-bearing mouse treated with vvDD-IL2 for ~12 h. The flow cytometry analysis for IFN-γ^+ $CD8^+$ T cells suggested that fractions of TILs were able to recognize p15E and B8R epitopes, respectively (Supplementary Figure 2).

OV-induced TILs can be isolated from tumor tissues and expanded *ex vivo*

As our eventual aim was to utilize these tumor-reactive TILs for therapeutic ACT, we investigated the ability to expand these TILs while retaining their tumor-specificity. First, we established protocols for TIL isolation with CD90.2 magnetic beads, and then for *ex vivo* culture and expansion. For induction of tumor-specific TILs, MC38 s. c. tumor-bearing mice were treated as before with intratumoral PBS or vvDD-IL2. Ten days later tumors were harvested and TILs from each tumor were cultured in the presence of IL-2 and IL-7 in RPMI complete media for 3 days. To test if the cultured TILs retained their tumor specificity, these T cells were tested for their tumor recognition using a co-culture assay including irradiated MC38 tumor cells and irrelevant target cells -γ-irradiated B16 tumor cells or naïve splenocytes from non-tumor-bearing B6 mice. After 24 h these T cells were analyzed for tumor specificity using IFN-γ ELISpot or 4–1BB expression by flow cytometry. According to the IFN-γ ELISpot assay, T cells expanded from vvDD-IL2 or PBS-treated tumors demonstrated increased MC-38 specific IFN-γ secretion compared to irrelevant target cells (B16 or naïve splenocytes) (Fig. 4A; Suppl. Fig. 3). To distinguish between $CD8^+$ and $CD4^+$ tumor-specific T cell response, flow cytometry results of 4–1BB expressing $CD8^+$ and $CD4^+$ T cells from each expanded TIL culture are summarized in Fig. 4 (B, C) and Suppl. Fig. 3. The expanded cultures from the vvDD-IL2-induced TILs demonstrated a higher percentage of tumor-specific $CD8^+$ and $CD4^+$ T cells when compared to PBS treatment [% $CD8^+$ $4–1BB^+$ T cells: vvDD-IL2: 16.4 ± 11; PBS: 1.96 ± 1.6. % $CD4^+$ $4–1BB^+$ T cells: vvDD-IL2: 6.3 ± 2.4; PBS: 2.4 ± 2.0].

ACT of OV-induced TILs led to therapeutic efficacy in a low-immunogenic colon tumor model

We then explored the ability of the vvDD-IL2-induced TILs to be utilized for ACT for cancer treatment. The overall schema is shown in Fig. 5A. We harvested the TILs from MC38 colon cancer-bearing mice treated with vvDD-IL2 as described in Methods. As a

control, T cells were isolated from splenocytes from naïve mice. These TILs and control T cells were expanded *ex vivo* for 4 days as described above and then portions of the cultures were characterized. Expanded TILs from vvDD-IL2 injected tumors had a higher specific reactivity against MC38 compared to naïve splenocytes expanded in an identical manner (Fig. 5, B & C). No IFN-γ was released when cultured control T cells were tested against MC38 cancer cells. We also tested the activation status of the vvDD-IL2-induced TILs using 4–1BB staining and found that 6% of the $CD8^+$ T cells and 5% of $CD4^+$ T cells were 4–1BB positive after co-culture with irradiated MC38 tumor cells, as analyzed by flow cytometry (Fig. 5, D & E; Suppl. Fig. 4). Very low levels of 4–1BB were found when these TILs were co-cultured with B16 tumor cells, naïve splenocytes or growth medium. These results indicated that $CD4^+$ and $CD8^+$ TILs specifically recognized irradiated MC38 cancer cells after expansion.

We next examined the therapeutic efficacy of the *ex vivo* expanded TILs via ACT in a peritoneal MC38 colon cancer model (Fig. 6). Seven days after inoculation of 5.0e5 MC38-luc cells i.p. into C57BL/6J mice, the tumor growth was monitored by bioluminescence imaging using the Optical *In Vivo* Imaging System, and mice were randomly divided into groups with an equivalent range of tumor burden. Prior to ACT, tumor-bearing mice received 5 Gy of sublethal irradiation to mimic lymphodepletion similar to clinical protocols. The mice were injected i.p. with vvDD-IL2-induced and *ex vivo* expanded TILs, *ex vivo* expanded control T cells, or PBS saline. All treated mice received exogenous cytokine support with recombinant IL-2. We then followed the therapeutic response by live animal bioluminescence imaging to monitor the kinetics of tumor growth over time (Fig. 6A, B). It is clear that by day 17 post ACT, the group treated with ACT with T cells from vvDD-IL2 mice and radiation showed the best results ($p < 0.05$, compared to any other group). Mice treated with ACT from vvDD-IL2-induced TILs showed the least amounts of tumor burden and survived the longest when compared to PBS or 5Gy/IL-2 controls ($p < 0.001$) (Fig. 6C). If we compare the median survival of mice in different groups, the ACT with T cells from VV-IL2 group gave the very best result, with median survival time at 61 days (Fig. 6D; $p < 0.01$, compared to any other group).

DISCUSSION

ACT using autologous TILs represents a personalized cancer immunotherapy strategy, targeting shared and unique tumor antigens expressed by a patient's tumor [2, 34]. ACT for cancer using patients' own TILs has been a successful systemic treatment leading to the cure of a number of advanced melanoma patients, and a few other tumor types. The suppressive TME is addressed with a preparatory non-myeloablative chemotherapy regimen (cytoxan and fludarabine) then *ex vivo* expanded TILs are delivered intravenously followed by IL-2 to maintain T cell activity. A recent study in uveal melanoma demonstrated a strong association between the anti-tumor reactivity of the infused TILs and objective clinical response. Thus, isolation and delivery of TILs with greater anti-tumor reactivity may result in improved clinical responses. Discovering new methods to improve the recovery of tumor-reactive TILs could have a significant impact on the field of cancer immunotherapy and ultimately patient outcomes.

Immune cell infiltration in the tumor impacts tumor progression and patient survival, and a strong lymphocyte infiltration has been reported to be associated with an antitumor response and improved clinical outcome in a variety of types of cancers, including colorectal cancer [35–39]. The majority of solid tumors, especially those of low tumor mutational burden (TMB), however, lack an inflammatory infiltrate. Cancers develop immune escape mechanisms to avoid detection by effector immune cells. This includes cell surface expression of immune system checkpoint ligands such as PD-L1 [40, 41]; secretion of soluble immunosuppressive factors such as transforming growth factor beta, vascular endothelial growth factor, interleukin-10, galectin-1, indoleamine 2,3-dehydrogenase [42–44]; down-regulation of major histocompatibility complex (MHC) class I expression; and overexpression of receptors such as C-X-C chemokine receptor type 4, basic fibroblast growth factor and epidermal growth factor [45, 46]. The immunosuppressive tumor microenvironment includes immunosuppressive macrophages, myeloid-derived suppressor cells and regulatory T cells interfering with an efficient anti-tumor T cell response. Inhibitory checkpoint molecules such as CTLA-4, PD-1, TIM-3, LAG3, are upregulated in chronically stimulated T cells, promoting T cell anergy.

OVs overcome the immunosuppressive TME as they turn cold tumor ‘hot’ [8–10]. They infect and kill tumor cells *in vivo* and induce an inflammatory response that clears the virus and some non-infected tumor cells, despite the immunosuppressive TME. The virus is ultimately immunologically cleared from the tumor and tumor reactive T cells are generated. This local cytotoxic and anti-tumor immunologic activity has limited systemic activity. Successful systemic delivery of OVs with adequate tumor infection throughout the body for a meaningful effect on metastatic tumors, remains a challenge. One way to take advantage of local anti-tumor immunity for systemic therapy is to harvest the infected tumor tissues, isolate and expand the tumor-reactive T cells *ex vivo*, and adoptively transfer them back into the patient. In a clinical study done by Ribas et al., intratumoral injection of talimogene laherparepvec in melanoma patients resulted in an increase in infiltrating T cells independent of the T cell baseline [9]. In our study, we have demonstrated for the first time the feasibility of using these TILs for therapy. When the subsets of T cells in the TIL populations were analyzed, the quality of TILs was much improved in the mice treated with vvDD. They contained much higher percentages of T*cm* and T*em* cells. Moreover, vvDD-IL2 improved the quality even further, as it contained fewer exhausted PD-1^{hi}Tim-3^{+} $CD8^{+}$ T cells and T*reg* cells.

This would effectively expand the utility of both TILs and OV. This immunotherapy would be personalized for each patient’s tumor neoantigens, and in contrast to CAR-T cells would be polyclonal in terms of antigen recognition and immune cell type and unlikely to have reactivity against normal cells (off target toxicity).

Our laboratories have extensive experience with both OVs and T cell-mediated immunotherapy [47, 48]. The anti-tumor immune response induced by oncolytic VV has been studied extensively. Previous depletion studies in animal models indicated the anti-tumor response and long-term survival are attributed to $CD8^{+}$ T cells, and to a smaller extent $CD4^{+}$ T cells, NK cells and neutrophils [8, 26, 49]. In the current study, we compared the parental oncolytic VV (vvDD) with those expressing various cytokines and chemokines

(IL-2, CCL5, CXCL11 and IL-15) for their capacity to elicit and attract tumor-reactive TILs. Under the experimental conditions, we found that vvDD-IL2 performed the best, leading to the highest levels of tumor-reactive $CD8^+$ TILs. The virus induced a MC38-specific T cell response and the effect was not reproducible by injection of the cytokine without the virus. The cytokine IL-2 was approved to treat metastatic melanoma and renal cancer in the 1990s. Since then different application strategies and combinations have been tested to overcome toxicity and enable a specific anti-tumor immune response [50, 51]. Although the transfer of virus-specific peripheral blood T cells to treat cancer patients have been done [52, 53], our current study has demonstrated, for the first time, a new therapeutic method-ACT using virally induced TILs isolated from solid tumors. The data from immunostaining confirmed that vvDD-IL2 attracts high numbers of total $CD3^+CD8^+$ T cells in the tumor with an increased percentage of specific tumor reactivity.

These TILs isolated from tumors treated with vvDD-IL2 were able to be expanded in culture and maintain their activity and specificity. We observed high levels of IFN-γ release and 4–1BB expression from $CD8^+$ and $CD4^+$ TILs after 4 days of *in vitro* expansion when co-cultured with the same tumor cells. Adoptive transfer of these TILs in a model of peritoneal metastases reduced tumor burden and achieved better survival compared to the control groups. Thus, we have demonstrated the principle of therapeutic efficacy for OV-elicited tumor-reactive TILs in a murine tumor model.

Virally induced tumor-reactive TILs have not previously been tested in ACT for therapeutic effectiveness. In the literature, most studies on TILs have been performed in patients by culturing small pieces of the tumor and expanding the outgrowing T cells [2]. It is well known that isolation of murine TILs is challenging and there exist only a few reports documenting the culturing of murine TILs [54, 55]. Most ACT studies in the murine system have been conducted using T cells derived from splenocytes and universally activated with super-antigens (such as anti-CD3 and CD28 antibodies). A number of investigators have explored the combination of an OV with ACT in cancer models, using the virus to improve trafficking of adoptively transferred cells [56–58], but not as a “pre-TIL” approach to improve the recovery of tumor-reactive T cells. Murine TILs have been isolated with magnetic beads or FACS sorting from tumor tissue-derived single cell suspensions [29, 54, 55]. We utilized magnetic bead sorting (CD90.2 antibody) for isolation, and murine TILs were able to be expanded *ex vivo* only when clearly separated from the tumor tissue. When assayed for their functionalities after *ex vivo* short-term expansion they retained their tumor specificity. In our studies MC38 tumor specific 4–1BB expression was enhanced in about 5–20 % of the $CD8^+$ T cells and about 5–6 % of the $CD4^+$ T cells.

We are fully aware that the murine system for *ex vivo* TIL expansion has limitations and that the i.p. tumor serves only as a model to prove the therapeutic principle. In the future, we will explore the intravenous delivery of TILs to solid tumor models as the homing potential of such *ex vivo* expanded TILs needs to be analyzed as well. We would like to explore ways to induce, isolate, and expand more optimized TILs for ACT using OVs. For example, it is known that stem cell-like T cells may offer more therapeutic potential [59, 60]. Our comparison of cytokines and chemokines have not been exhaustive, and other agents may have similar or more potent activity as a “pre-TIL” approach. Once these types of questions

are addressed, it will be time to translate this improved approach and technology from bench to bedside. In addition, one key issue in ACT is the trafficking of TILs intravenously delivered back to the tumor tissues where they exert their key functions. The study on trafficking of TILs back to tumor tissue is out of scope of this study as many other studies have been done to find the right answers [61].

In summary, we have demonstrated that IL-2 armed OV promotes T cell infiltration and enhances the population of tumor-reactive TILs in a murine colon cancer model. Our report presents a new therapeutic strategy to promote the generation and infiltration of tumor-reactive TILs in lowly or poorly immunogenic tumors, and the expansion of such TILs for ACT. The new strategy may be translated into a clinical application and may allow adoptive T cell therapy for an expanded group of cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the University Imaging Core Facility for technical assistance in immunostaining and imaging, the Hillman Cancer Center Biostatistics facility for consultation, and Prometheus (San Diego, CA) for human recombinant IL-2 used in the study.

Funding

This work was supported in part by David C. Koch Regional Cancer Therapy Center. MF (DFG research fellowship 1655/1-1) and EG were supported by fellowships from German Research Foundation. ED was supported by fellowships from the China Scholarship Council, China. This project has used the University of Pittsburgh shared facilities (Animal Facility, Genomics Research Core, Flow Cytometry) that are supported in part by the NIH grant award P30CA047904.

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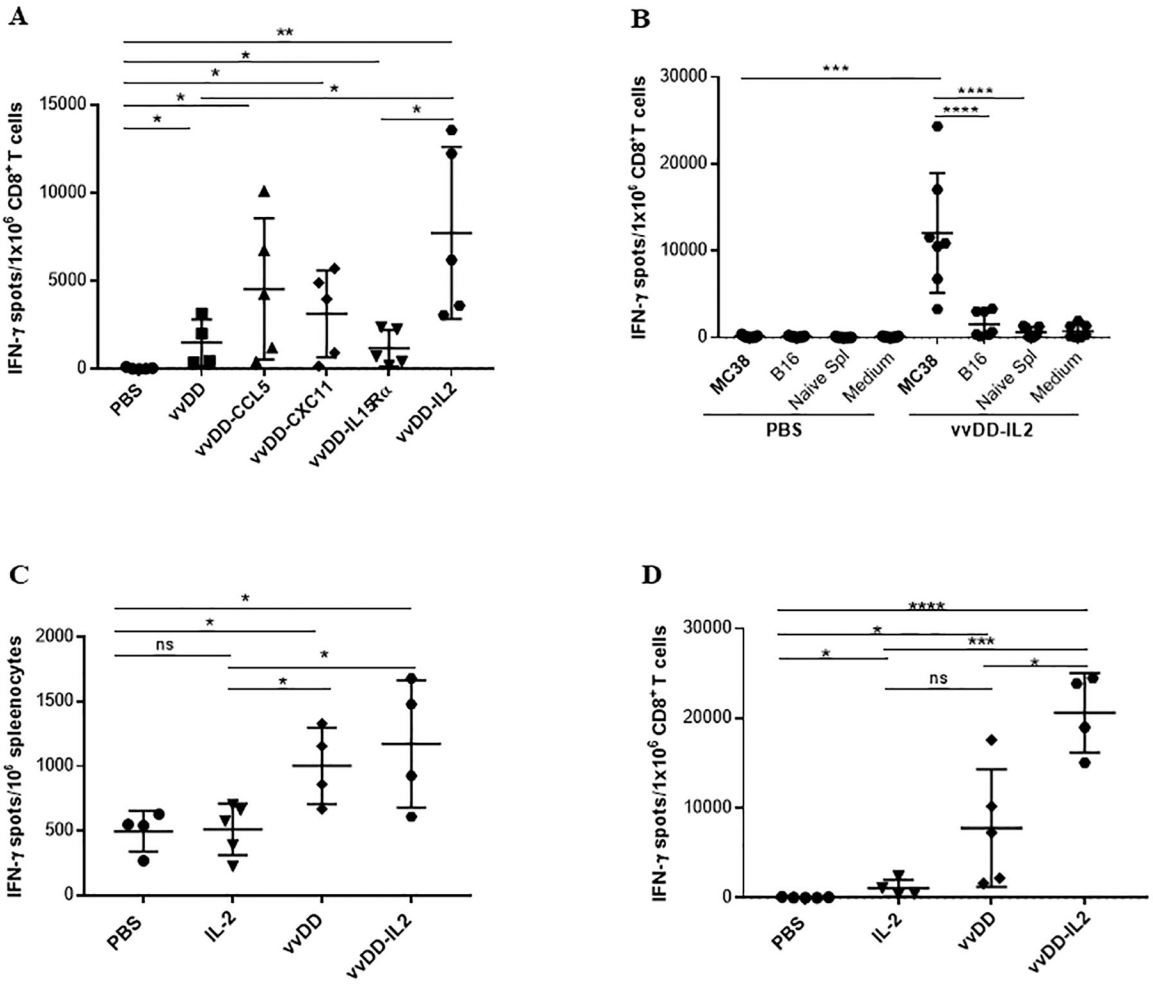

Figure 1. Oncolytic VVs elicit tumor-specific antitumor CD8$^+$ T cell response in the tumor tissue.

Ten days after viral treatments, subcutaneous MC38 tumors and/or splenocytes were collected and single cell suspensions were made followed by magnetic separation. Then isolated CD8$^+$ T cells or splenocytes were tested by IFN-γ ELISPOT assay. (**A**). CD8$^+$ T cells isolated from tumors (n = 4 – 5 mice/group) were either left unstimulated or challenged with γ-irradiated MC38 tumor cells for 24 h. Results were shown as individual data points (number of spots in each well) and bars (means ± standard deviation) of IFN-γ$^+$ CD8$^+$ T cells from each mouse evaluated in triplicate. Data from one experiment representing 2 independent experiments are shown. (**B**). Tumor-specificity of the OV-induced CD8$^+$ TILs (n = 7 mice/group). Data are from one experiment representative of 3 independent experiments. For multiple group comparison, One-way ANOVA was used. ***$p < 0.001$; ****$p < 0.0001$. (**C**). MC38 tumor cell reactivity of splenocytes by IFN-γ ELISPOT assay. Splenocytes from treated mice were either left unstimulated or challenged with γ-irradiated MC38 tumor cells for 24 h. (**D**). MC38 tumor cell reactivity of isolated CD8$^+$ TILs by IFN-γ ELISPOT assay. One experiment representative of 2 independent experiments is shown (n = 4–5 mice/group). Student's t-test was used to analyze the statistical significance for data presented in panels A, C and D. *$p < 0.05$; **p < 0.01; ***$p < 0.001$; ****$p <$ 0.0001.

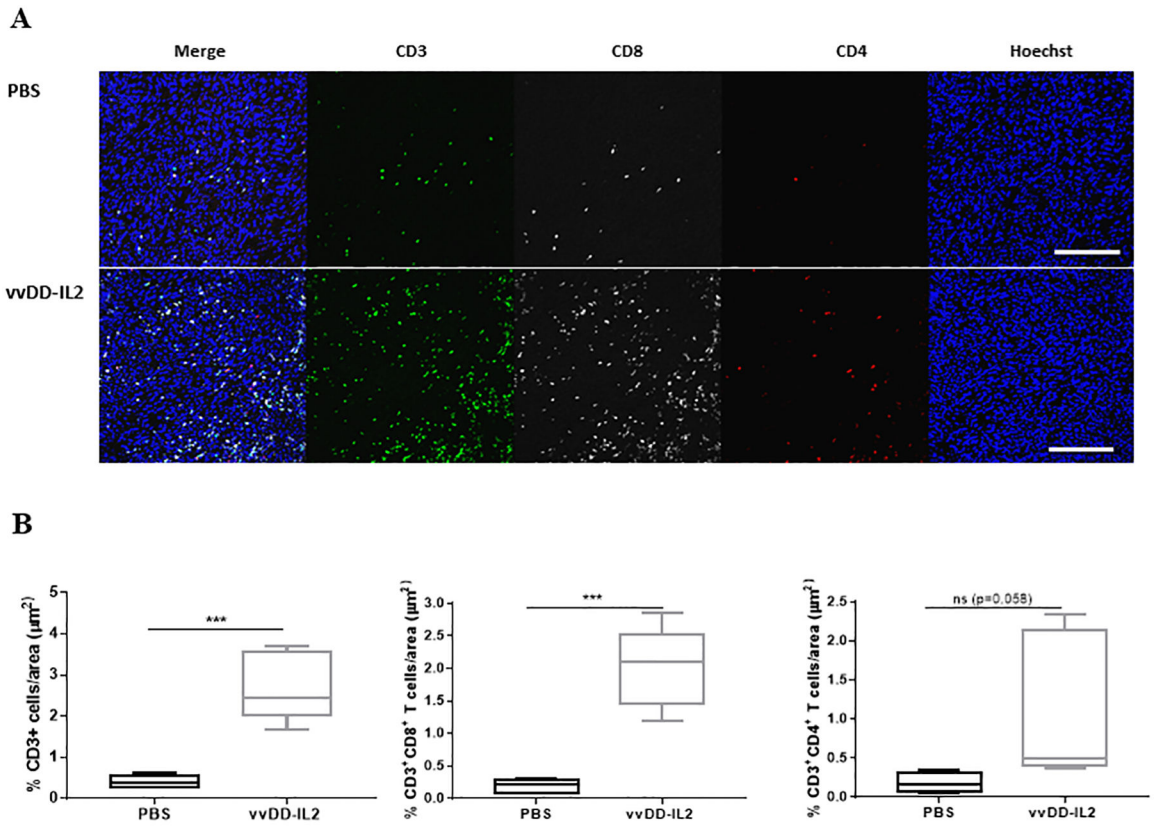

Figure 2. vvDD-IL2 treatments increase the number of TILs.

The tumor cell inoculation and tumor harvest were as described previously. Tumors were collected, fixed and stained for Hoechst (blue), CD3 (green), CD4 (red) and CD8 (white). **A**. Representative immunofluorescence image of one sample from each group (n = 5). For the representative panel, a single representative field was cropped from the original nine field acquisition. Scale bar 25 μm. **B**. Summary of the percentage of $CD3^+$ T cells and $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells per area. Student's t-test was used to analyze the statistical significance. ***$p < 0.001$.

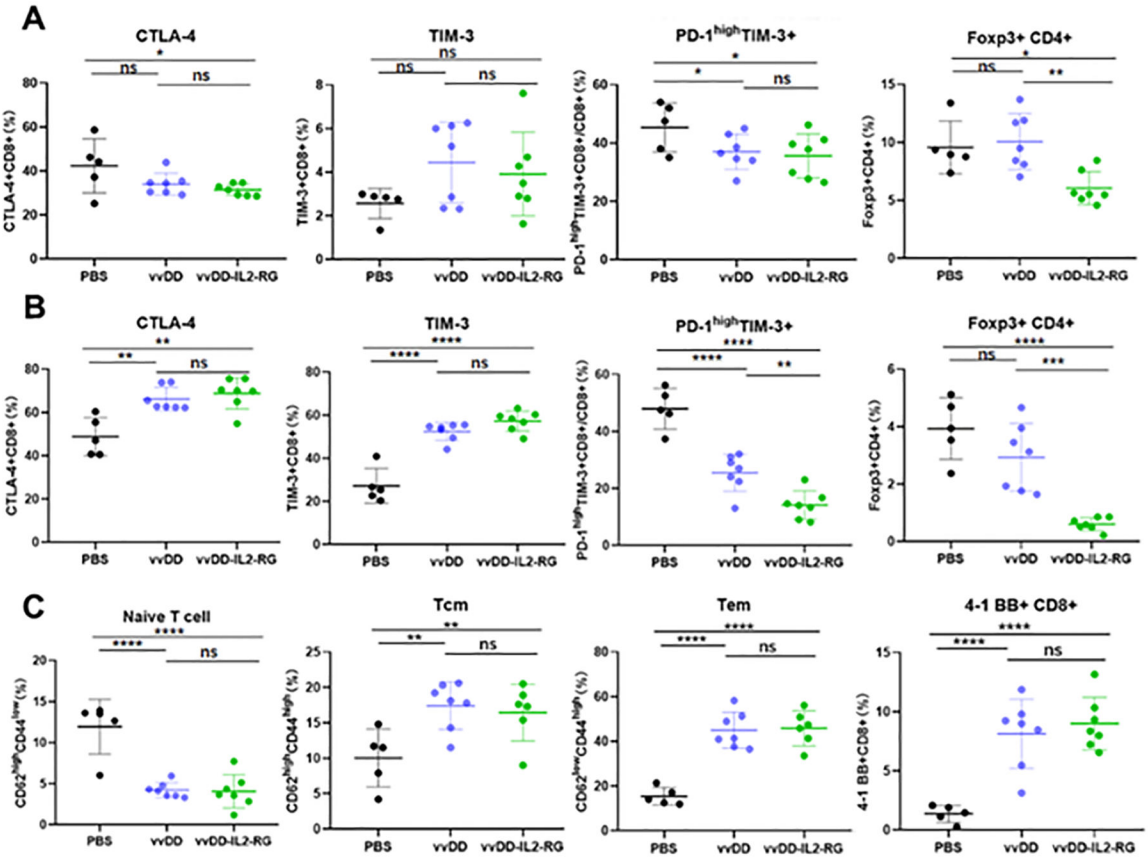

Figure 3. Immune status in the TME and spleen post virus treatment.

B6 mice were inoculated subcutaneously with 5.0e5 MC38 tumor cells. When tumors reached 5 × 5 mm (about day 9) they were treated intratumorally with PBS, vvDD, vvDD-IL-2 at a dose of 1.0e8 PFU. The mice were sacrificed 10 days post viral treatment and primary tumors and splenocytes were collected and analyzed by flow cytometry to determine the activation and immunosuppressive markers in (A) splenocytes, (B) TILs and (C) subsets of $CD8^+$ T cells in the TILs. Tn for naïve T cells; Tcm for central memory T cells, and Tem for effector memory T cells. The percentages are over total $CD8^+$ T cells, or over total $CD4^+$ T cells for T*reg* cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$.

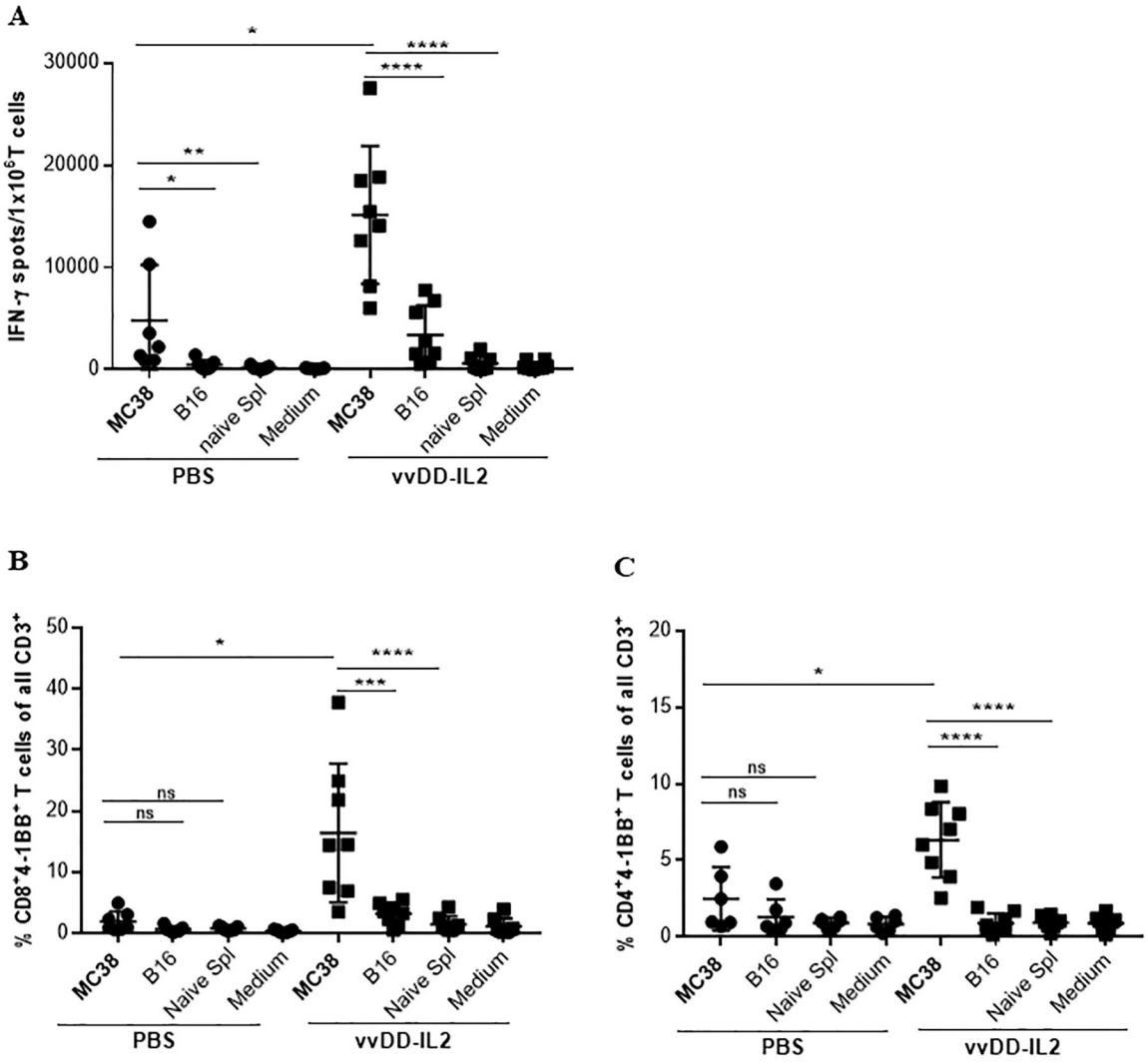

Figure 4. OV-induced TILs can be cultured and expanded *ex vivo* and retain their tumor specificity.

(**A**). Tumor specificity of the expanded TILs. TILs from each individual mouse have been cultured for 4 days and then tested by IFN-γ ELISPOT assay. (**B, C**). Analysis of 4–1BB upregulation on (B) $CD4^+$ and (C) $CD8^+$ T cells by flow cytometry. As previously described, T cells were either left unstimulated (medium) or challenged with γ-irradiated MC38 tumor cells or γ-irradiated B16 tumor cells or naïve splenocytes from non-tumor-bearing B6 mouse in duplicate. After 24 h the cells have been stained for flow cytometry analysis for CD3, CD4, CD8, 4–1BB. Results are shown as individual data points (percentage of $CD8^+4$–$1BB^+$ T cells and $CD4^+4$–$1BB^+$ T cells) and bars (means ± standard deviation) of T cells from each mouse evaluated in duplicate. Data are presented as summary from 2 out of 5 independent experiments (n = 3–4 mice/group). For multiple group comparison One-way ANOVA was used. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

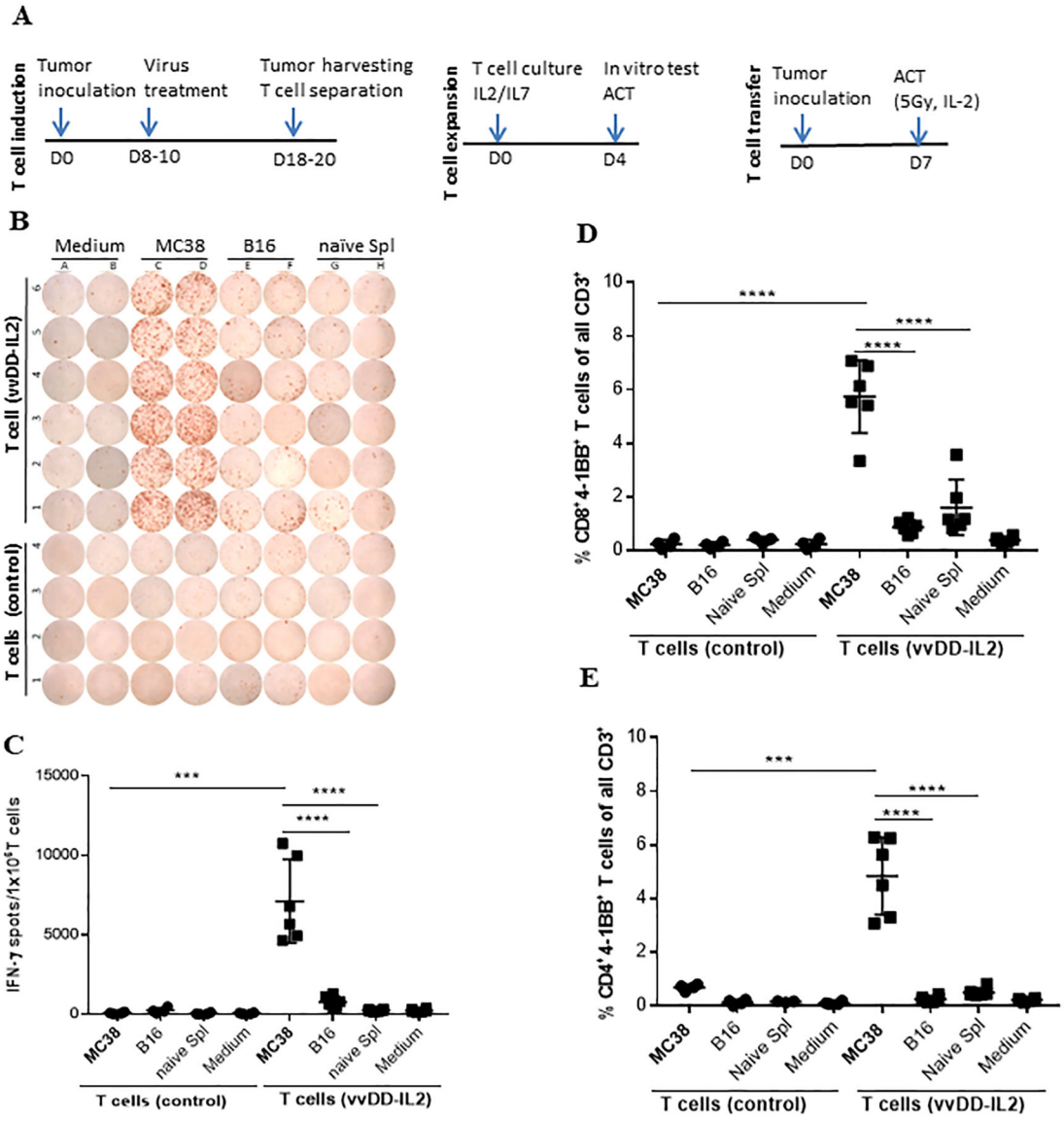

Figure 5. Analysis of the vvDD-IL2 induced TILs after *ex vivo* expansion.

Prior to ACT, samples (T cells from naïve spleens: n = 4; TILs: n = 6) of the *ex vivo* cultured and expanded TILs were tested for tumor reactivity using IFN-γ ELISpot assay and co-culture assays for 4–1BB expression analyzed by flow cytometry as described previously. (**A**). Schema of experimental procedure. (**B, C**). Shown are (B) the ELISpot plate and (C) calculated IFN-γ^+ spots per 1.0e6 T cells. **(D, E)**. The cultured and expanded TILs were analyzed for cell surface 4–1BB expression of (D) $CD4^+$ and (E) $CD8^+$ T cells by flow cytometry. For multiple group comparison One-way ANOVA was used. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

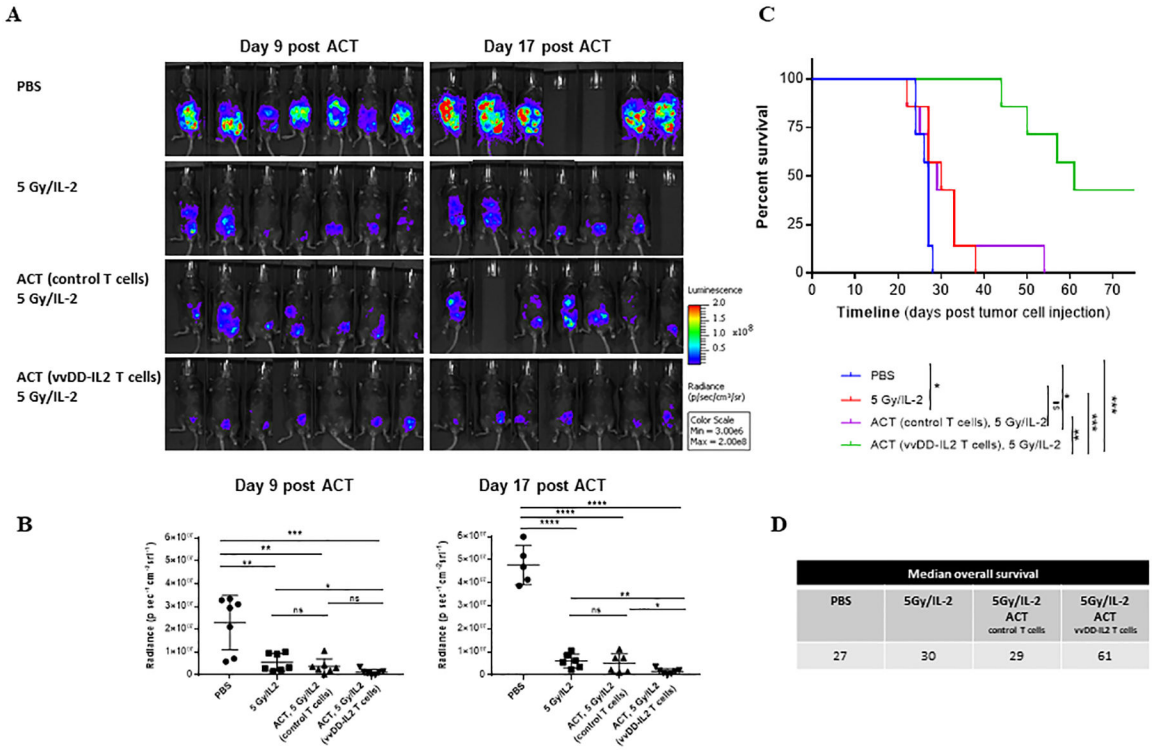

Figure 6. ACT of oncolytic virus-induced TILs led to significant therapeutic efficacy in mice bearing peritoneal carcinomatosis of MC38 colon cancer.

B6 mice were intraperitoneally inoculated with 5×10^5 MC38-luc cancer cells followed in 7 days, these mice were imaged for tumor growth and randomly grouped (n = 7 mice/group). Prior to ACT, mice in the treated groups received 5 Gy of sublethal irradiation. Grouped mice were intraperitoneally injected with PBS, naïve T cells, or vvDD-IL2 induced and *ex vivo* expanded TILs. All treated mice received exogenous IL-2 (1.0e5 IU/mouse, i.p. injection once every ~12 h for 3 days). One experiment representative of two independent experiments is shown (**A**, **B**). (A). Tumor growth has been monitored by live animal imaging 9 days and 17 days post treatment. (B). Radiance data quantified for two time points at days 9 and 17 post ACT. Student's t-test was used to analyze the statistical significance. The variance was similar between the groups in this experiment. (**C**). The long-term survival of tumor-bearing mice was monitored by Kaplan-Meier analysis. These data represent one of the two independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (D). Summary of median survival in the different treatment groups. This experiment was performed 2 times.