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BRIEF REPORT

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Synergistic antitumor response with recombinant modified virus Ankara armed with CD40L and CD137L against peritoneal carcinomatosis

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ABSTRACT

Recombinant-modified vaccinia virus Ankara (rMVA) is known to elicit potent antitumor immune responses in preclinical models due to its inherent ability to activate the innate immune system and the activation of adaptive responses mediated by the expression of tumor antigens and costimulus-providing molecules, such as CD40L and CD137L. Here, we evaluated different rMVA vectors in preclinical peritoneal carcinomatosis models (ID8.OVA-*Vegf*/GFP and MC38). We compared rMVA vectors expressing a tumor antigen (OVA or gp70) either alone or co-expressed with CD40L or/ and CD137L. In tumor-free mice, the vector coding for the triple combination was only slightly superior, whereas, in tumor-bearing animals, we observed a synergistic induction of T lymphocytes specific against vector-encoded and non-encoded tumor-associated antigens. The enhanced activation of the immune response was associated with improved survival in mice with peritoneal carcinomatosis treated with a rMVA vector encoding both CD40L and CD137L. Thus, the triple transgene combination in vaccinia viral vectors represents a promising strategy for the treatment of peritoneal carcinomatosis.

Introduction

Peritoneal carcinomatosis represents an advanced stage of gynecological and gastrointestinal cancer characterized by the invasion of the peritoneal cavity lining by malignant cells.[1] The highest incidence of peritoneal carcinomatosis is observed among patients with ovarian and colorectal cancer.[2,3] This advanced stage of peritoneal carcinomatosis is associated with a dismal prognosis, and the limited therapeutic options do not significantly impact the survival of the patients.[3,4] To address this unmet medical need, novel therapeutic strategies are being evaluated in clinical trials including intracavitary approaches.

Cancer vaccines are intended to expand tumor antigen-specific CD4+ and CD8+ T lymphocytes. Still, the impact on the overall survival of current vaccines has been limited, and the only cancer vaccine approved by the FDA has been sipuleucel-T for the treatment of prostate cancer, which provides a median overall survival advantage of approximately 4 months.[5] Several advances in the field hold promise for the next-generation vaccines. First, the advent of the anti-PD-(L) 1 monoclonal antibody provides a means to unleash the antitumor potential of the vaccine-expanded T lymphocytes.[6] Second, tumor neoantigens represent a pool of epitopes that evade the central tolerance mechanisms, and personalized antitumor vaccine based on tumor neoantigens has yielded promising results in phase I clinical trials.[7] Third, the expression of co-stimulatory molecules and the tumor antigen might provide the required signals to ensure the optimal expansion, differentiation, and persistence of tumor-specific T lymphocytes.[8–10] Finally, live viral vaccine vectors induce the release of danger- and pathogen-associated molecular patterns that provide adequate context for the activation of antigen-presenting cells at the same time, that they uptake and present tumor antigens expressed by the viral vectors.[11] Modified vaccinia virus Ankara (MVA) is a replication-deficient strain in human cells and potent inductor of type I interferon. The large cloning capacity of the vectors allows for several transgenes and, therefore, the simultaneous expression of tumor antigens and co-stimulatory molecules. Safety and immunogenicity have been demonstrated in clinical trials, and the FDA has approved this vector as a non-replicating vaccine against smallpox and monkeypox.[12]
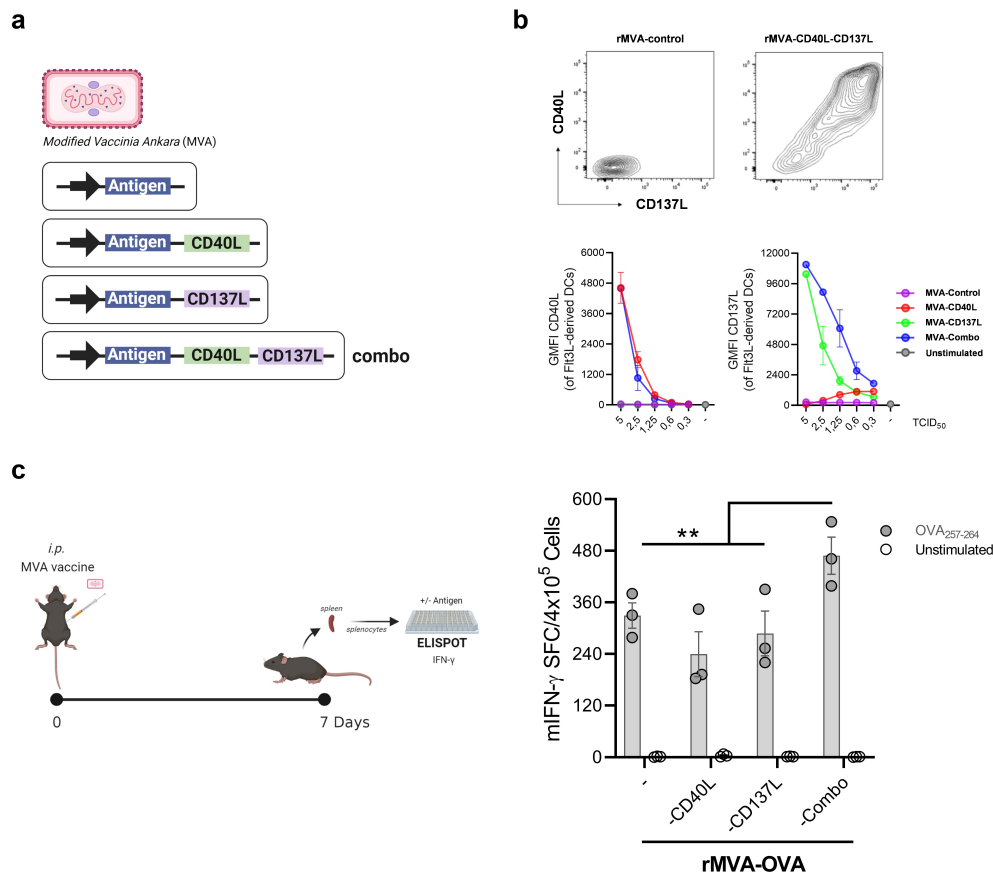
Figure 1. *In vitro* and *in vivo* rMVA characterization. (a) Schematic representation of rMVAs constructs. (b) Flow cytometry analysis of Flt3L-derived DCs infected for 18 h with rMVA-Control, rMVA-CD40L, rMVA-CD137L or rMVA-CD40L and rMVA-CD137L (rMVA-Combo). Geometric mean fluorescence intensity (GMFI) of CD40L and CD137L is shown. (c) OVA-specific IFN-γ–producing cells measured by ELISpot seven days after immunization of C57BL/6 mice (*n = 3*) with rMVA-OVA, rMVA-OVA-CD40L, rMVA-OVA-CD137L or rMVA-OVA-Combo *i.p.* administration. Data are represented as mean ± SEM. Two-way ANOVA followed by Sidak's posttest. *$p < .05$. *i.p*, intraperitoneal; MVA, modified vaccinia virus Ankara; OVA, ovalbumin.

Recently, the expression of CD40 ligand or CD137 ligand with tumor-associated antigens by MVA vectors has been shown to enhance the antitumor efficacy of these vectors dramatically.[13,14] CD40 plays a critical role in the activation of dendritic cells. Ligation of CD40 on the professional antigen-presenting cells triggers the production of IL-12 and licenses them for CD8^{+} T lymphocyte priming.[15,16] CD137 is expressed in activated T lymphocytes, and its ligation promotes survival, expansion, and effector functions of activated T cells.[17,18] Thus, CD40 and CD137 are potent immunostimulatory molecules with complementary mechanisms of action. The potential of combining both co-stimulatory pathways has been previously demonstrated in preclinical cancer models.[19]

In this study, we evaluated rMVA vector encoding tumor-associated antigens alone or co-expressed with CD40L, CD137L, or both (Combo) in two models representing ovarian and colon cancer peritoneal carcinomatosis. In these difficult-to-treat settings, the rMVA vector encoding tumor-associated antigens alone was inefficient, and the best results in terms of immune response activation and antitumor effect were achieved when both CD40L and CD137L were co-expressed along with tumor-associated antigens.

Results and discussion

Characterization of rMVAs encoding tumor antigens alone or in conjunction with CD40L and/or CD137L

In order to study the potential synergistic activity of co-expressing CD40L and CD137L in rMVA vectors for the treatment of peritoneal carcinomatosis, rMVAs encoding different combinations of tumor-associated antigens and co-stimulatory ligands were constructed and produced. These vectors conferred expression as a surrogate (ovalbumin – OVA-) or a *bonafide* tumor antigen (gp70) either alone or combined with CD40L and/or CD137L (rMVA-Combo) (Figure 1(a)). *In vitro*, these vectors infected efficiently Flt3L-derived mouse DCs, and the transgene-encoded co-stimulatory ligands were readily detected by flow cytometry. The expression levels of CD40L and CD137L exhibited a clear dose-response relationship (Figure 1(b)). In tumor-free animals, the intraperitoneal administration of all different rMVAs encoding OVA induced a potent adaptive response as measured by the OVA-specific IFN-γ production by T lymphocytes 1 week after vaccination. Interestingly, the co-expression of CD40L or CD137L alone did not enhance the OVA-specific IFN-γ release induced by the rMVA-OVA, illustrating the potent adjuvant activity of the

vector. This response was improved by vector encoding both CD40L and CD137L, which significantly increased the OVA-specific T-cell immune response (Figure 1(c)).

Loco-regional intracavitary treatment with rMVA-Combo elicits a potent effector T-cell activity and epitope spreading in models of peritoneal carcinomatosis

We intraperitoneally (i.p.) challenged mice with the ovarian cancer cell line ID8.OVA-*Vegf*/GFP to study the efficacy of rMVA with OVA as antigen in the different constructs. After inoculating ID8.OVA-*Vegf*/GFP i.p., we treated such mice with PBS (control group), rMVA-OVA, rMVA-OVA-CD40L, rMVA-OVA-CD137L, or rMVA-OVA-Combo. Two vector doses were administered at 7 and 21 days after the inoculation of tumor cells. At 21 days after the first dose (day +28 post-peritoneal carcinomatosis challenge), we collected the spleens to stimulate the splenocytes with OVA$_{257-264}$ to study the production of IFN-γ by CD8$^+$ T cell (Figure 2(a-b)).

In contrast to the results obtained in tumor-free mice (Figure 1(c)), rMVA-OVA, rMVA-OVA-CD40L, rMVA-OVA-CD137L failed to activate an IFN-γ-mediated T-cell response. These results might indicate that the immunosuppressive mechanisms deployed by the presence of tumors in the peritoneal cavity abrogated the immunostimulatory effect of these vectors. Remarkably, in this aggressive tumor setting, the rMVA that encoded with both CD40L and CD137L was able to induce a potent effector T cell response with a drastic increase of IFN-γ secretion by antigen-specific CD8$^+$ T cells when compared to rMVA-OVA-CD40L or rMVA-OVA-CD137L (Figure 2(b)). Cells retrieved from peritoneal lavages were also tested to study their specific IFN-γ production. Similarly, the maximum effect was observed in the rMVA-OVA-Combo group, leading to saturation of the signal in the conventional ELISpot assays (**Supplementary Fig. 1**). Furthermore, the quantification of IFN-γ concentration by ELISA in the supernatant of these *in vitro* stimulated cells obtained from peritoneal washes showed a clear superiority of the vector co-encoding CD40L and CD137L for the induction of IFN-γ production upon antigen restimulation (Figure 2(c)). Next, we incubated the splenocytes from the different experimental groups with the parental cell line ID8-*Vegf*/GFP, which did not express OVA, to evaluate the possibility of epitope spreading toward endogenous tumor antigens. Once again, the response in the rMVA-OVA-Combo group was significantly higher than in any of the other treated groups as measured by IFN-γ induction (Figure 2(d)). To further validate this finding, we also evaluated the antigen-specific T-cell response in another peritoneal carcinomatosis model generated by the colorectal cancer cell line (MC38), in this case analyzing the response against the tumor associate antigen (TAA) glycoprotein 70 (gp70) (Figure 2(e)). The synergy of CD40L and CD137L as transgenes in the MVA vectors was confirmed in this model in terms of higher IFN-γ secretion (Figure 2(e)).

Intracavitary rMVA-Combo improves survival in mice bearing peritoneal carcinomatosis and enhances loco-regional antitumor immune responses

Because rMVA-Ag-Combo treatment showed improved *ex vivo* effector responses in terms of IFN-γ production from antigen-specific T cells, we evaluated the in vivo rMVA-Ag-Combo efficacy challenging mice with ID8.OVA-*Vegf*/GFP to study the effect on survival. In line with the observed enhanced immune responses, mice treated with rMVA-OVA-Combo showed improved survival compared to the rMVA-OVA group (Figure 3(a)). Of note, rMVA-OVA-CD40L and rMVA-OVA-CD137L did not show significant differences. However, with rMVA-OVA-Combo, a considerable improvement was observed. In addition, at day 76 (when the first mice died in the untreated group), we barely detected any mice with ascites (5%) in the group treated with rMVA-OVA-Combo, whereas a higher proportion of mice showed ascites in the rest of the groups. Ascites were determined as abdominal distention and indicated progression of peritoneal carcinomatosis in ID8 models (Figure 3(a)). The superiority of the rMVA encoding both immunostimulatory molecules was confirmed in the colon carcinoma MC38 model of peritoneal carcinomatosis. In this setting, the retrovirally encoded antigen gp70 was used as a tumor-associated antigen. Treatment of MC38 established in the peritoneal cavity with the vector rMVA-gp70-Combo significantly extended mice survival (**Supplementary Figure 2A**). Mice that fully eradicated the peritoneal tumors were rechallenged subcutaneously with MC38 cells 90 days after the first MC38 peritoneal inoculation. All *naïve* mice developed subcutaneous tumors, while mice that had previously eradicated peritoneal tumors were resistant to the rechallenge, indicating the development of immune memory (**Supplementary Figure 2B**).

In parallel to the survival experiment, we analyzed the kinetics of OVA-specific T lymphocytes in circulation in the groups treated with rMVA-OVA-CD40L, rMVA-OVA-CD137L, and rMVA-OVA-Combo treatments. rMVA-OVA-Combo shows a slight increase of tetramer-positive T cells compared to the other experimental groups after the first dose. Furthermore, differences increased after the second viral administration, indicating a better persistence of the effector immune response in the rMVA-OVA-Combo group (Figure 3(b)). We also analyzed the expansion of tetramer-stained lymphocytes in peritoneal lavages. The results showed a more significant loco-regional increment of tetramer-positive T lymphocytes following treatment with rMVA-OVA-Combo (Figure 3(c)).

One of the most relevant tissues where to study peritoneal carcinomatosis is the omentum. In fact, the omentum is considered to be the origin of peritoneal carcinomatosis, particularly in patients with ovarian cancer. Moreover, the omentum is a critical tissue for the development of immune responses in the peritoneum. In mice challenged with ID8.OVA-*Vegf*/GFP, the omentum growth correlated with the lethal progression of peritoneal carcinomatosis (Figure 3(d) and **Supplementary**
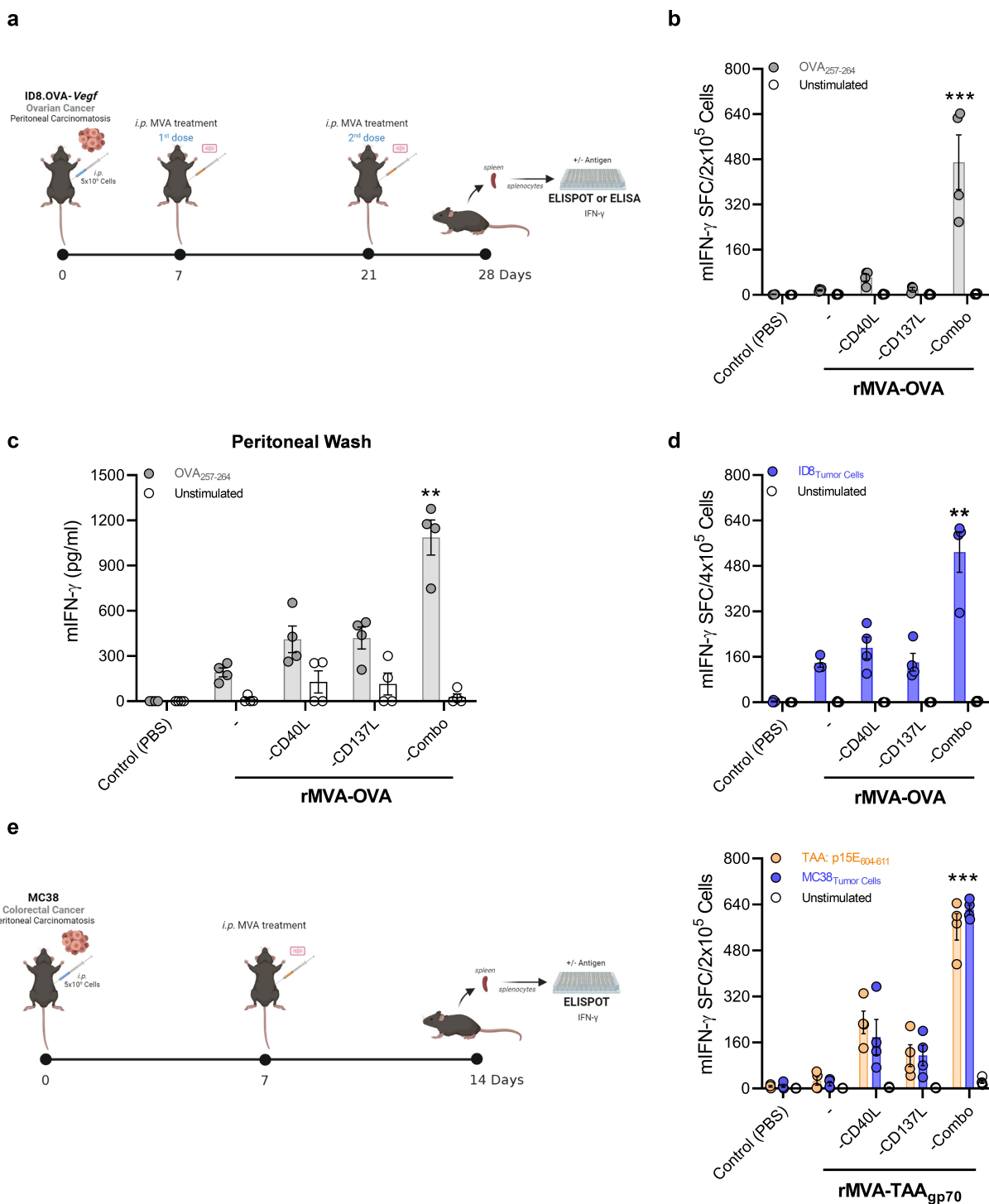
Figure 2. **rMVA-Combo induces a potent antitumor-specific immune response in peritoneal carcinomatosis models by intracavitary administration**. (a) C57BL/6 mice ($n = 4$) were challenged *i.p.* with 5×10^6 ID8.OVA-*Vegf*/GFP tumor cells. Seven days later, mice were treated by *i.p.* route with PBS (Control) or 5×10^7 TCID$_{50}$ of the indicated rMVAs constructs. A second dose (boost) of treatment was administrated twenty-one days after tumor cells inoculation. One week after the boost, splenocytes and peritoneal washes were collected. (b) IFN-γ–producing cells measured by ELISpot in splenocytes stimulated with the OVA$_{257-264}$ antigen. (c) IFN-γ–production measured by mouse IFN-γ ELISA in peritoneal washes stimulated with the OVA$_{257-264}$ antigen. (d) IFN-γ–producing cells measured by ELISpot in splenocytes stimulated with 1:10 irradiated (20,000 rads) ID8-*Vegf*/GFP tumor cells. (e) C57BL/6 mice ($n = 4$) were challenged *i.p.* with 5×10^5 MC38 tumor cells. Seven days later, mice were treated by *i.p.* route with PBS (Control) or 5×10^7 TCID$_{50}$ of the indicated rMVAs constructs. One week later, mice were euthanized and splenocytes were stimulated with p15E$_{604-611}$ antigen. IFN-γ–producing cells were measured by ELISpot. Data are expressed as mean ± SEM. Results are representative of two independent experiments. Two-way ANOVA was performed followed by Sidak's posttest. **$p < .01$; ***$p < .005$; *i.p*, intraperitoneal; MVA, modified vaccinia virus Ankara; OVA, ovalbumin; TCID$_{50}$, 50% Tissue Culture Infectious Dose.
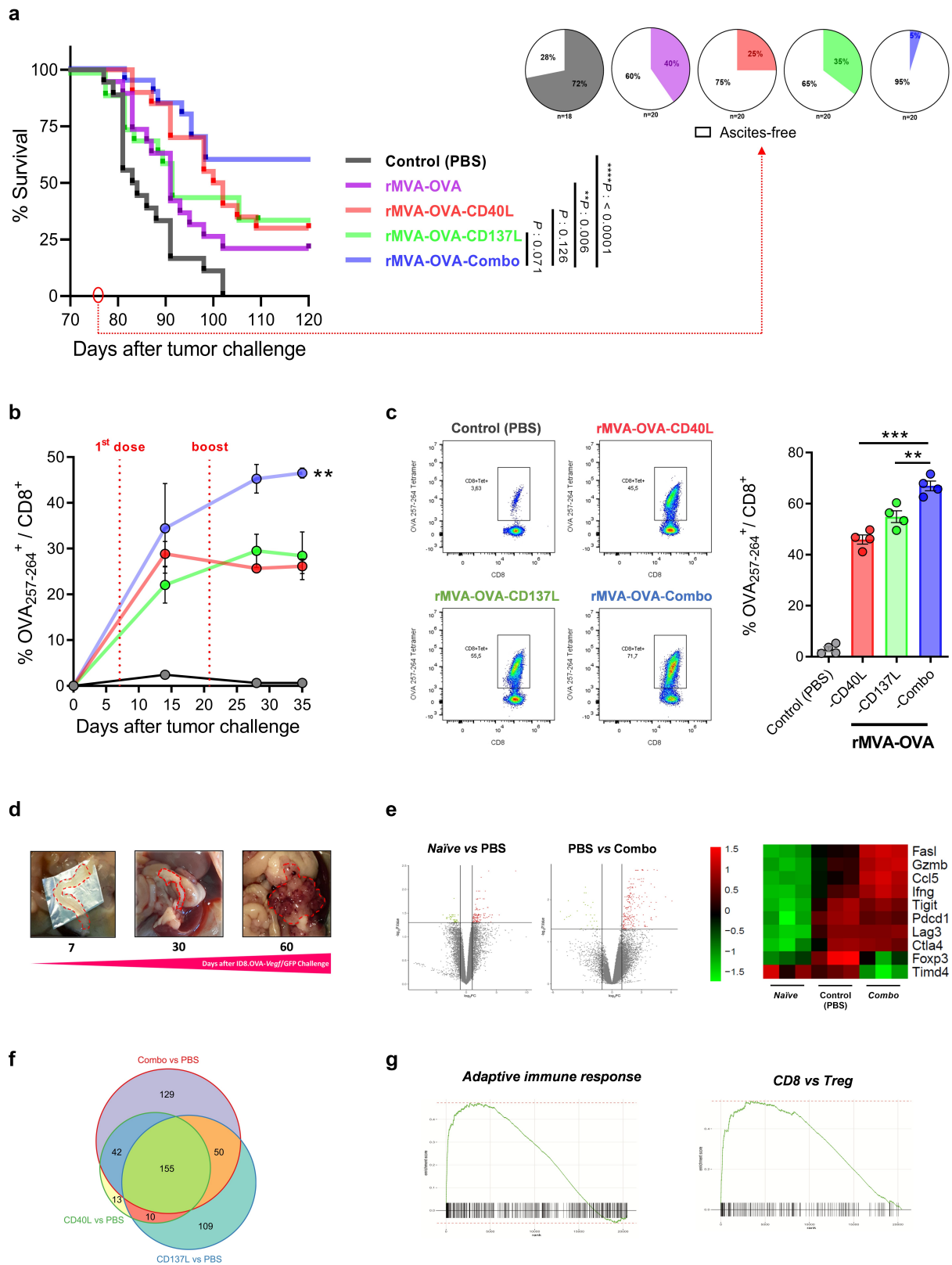
Figure 3. Loco-regional rMVA-Combo increases peritoneal carcinomatosis survival. C57BL/6 mice were challenged *i.p.* with 5×10^6 ID8.OVA-*Vegf*/GFP tumor cells. Seven and twenty-one days after tumor challenge, mice were treated by *i.p.* route with PBS (Control) or 5×10^7 TCID$_{50}$ of the indicated rMVAs constructs. (a) Kaplan-Meier survival curve and the percentage of ascites development at 76 days after peritoneal carcinomatosis challenge were represented ($n = 20$). (b) Percentage of OVA$_{257-264}$ Kb tetramer$^+$/CD8$^+$ cells 15, 28 and 35 days after tumor challenge in blood ($n = 3$), and (c) in peritoneal washes at 28 days after tumor challenge ($n = 4$). (d) Peritoneal carcinomatosis ID8.OVA-*Vegf*/GFP progression in the omentum. (e) Volcano plots and heatmap of *z*-scored log$_2$CPM expression of relevant genes in the tumor microenvironment identified by RNA-*seq* analysis in the omentum between the tumor treated with PBS (Control group) *vs.* non-tumor (*Naïve*), and rMVA-OVA-Combo *vs.* PBS. (f) Venn diagram representing the number of genes modulated in each experimental condition. (g) Pathways identified using Gene set enrichment analysis (GSEA). Data are expressed as mean ± SEM. Results are representative of two independent experiments. Two-way ANOVA was performed followed by Sidak's posttest. **$p < .01$; ***$p < .005$; *i.p*, intraperitoneal; MVA, modified vaccinia virus Ankara; OVA, ovalbumin; TCID$_{50}$, 50% Tissue Culture Infectious Dose.

Figure 3). This was confirmed by quantification of bioluminescence after intraperitoneal administration of ID8.OVA-*Vegf*/GFP.LUC (**Supplementary Fig. 3**). Finally, RNA-*seq* was performed in the omentum to determine the impact of our best therapeutic strategy at a molecular level. Differential gene expression was observed between the non-tumor (*Naïve*) *vs.* tumor-untreated or Control (PBS), and Control (PBS) *vs.* MVA-OVA-Combo group (Figure 3(e) and **Supplementary Figure 4**).

Interestingly, the RNA expression results showed that tumor inoculation induced an endogenous immune response dampened by several immunosuppressive mechanisms as reflected by high levels of *Foxp3, Lag3, Pdcd1, Ctla4*, and *Tigit*, among others (Figure 3(e)). rMVA-OVA-Combo significantly increased the expression of relevant genes that are crucial for the effector antitumor immune responses, such as cytotoxic molecules (*Ifng, Gzmb, FasL*) and chemokines responsible for T-cell recruitment (*Ccl5*), while downregulated immunosuppressive genes (*FoxP3, Timd4*) (Figure 3(e)). Interestingly, 129 genes were modulated exclusively by the rMVA-Combo, indicating a synergistic effect of the immunostimulatory molecules (figure 3(f)). Gene Set Enrichment Analysis (GSEA) also demonstrated that the treatment with rMVA-OVA-Combo upregulated a pathway of genes involved in the adaptive immune response and an inferred increase in the ratio CD8:Tregs (Figure 3(g)).

In conclusion, we have demonstrated that rMVA encoded tumor-associated antigen can be synergistically improved when CD40L and CD137L are co-expressed as molecules promoting T-cell costimulation. The MVA vector with the three transgenes elicited stronger immune responses in mice bearing peritoneal carcinomatosis that clearly improved survival, crucially inducing epitope spreading toward endogenous antigens absent from the vector.

Material and methods

Reagents and cell lines

Peptides OVA$_{257-264}$ and p15E$_{604-611}$ were synthesized by GeneCust (Boynes, France) and had a purity above 95% as determined by HPLC and mass spectrometry. Recombinants MVA-BN were developed by Bavarian Nordic and are deposited at the European Collection of Cell Cultures (V00083008). All recombinants were generated from a cloned version of MVA-BN in a bacterial artificial chromosome. Infectious viruses were reconstituted from bacterial artificial chromosomes by transfecting bacterial artificial chromosome DNA into BHK-21 cells and superinfecting them with Shope fibroma virus as a helper virus. After three additional passages of primary embryo fibroblasts, helper virus-free MVA recombinant viruses were obtained. All viruses used in animal experiments were purified twice through a sucrose cushion. ID8. OVA-*Vegf*/GFP, ID8-*Vegf*/GFP, and MC38 tumor cell lines were obtained from Dr. George Coukos (Ludwig Cancer Research, Switzerland) and Dr. Karl E. Hellström (University of Washington, Seattle), respectively. ID8.OVA-*Vegf*/GFP cells were grown in Dulbecco's modified Eagle's, high-glucose medium (Invitrogen, Carlsbad, CA) supplemented with 4% fetal

bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA), 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (Roche, Indianapolis, IN). MC38 cells were grown in Roswell Park Memorial Institute 1640 Medium with GlutaMAX supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM β-mercaptoethanol (Gibco, Thermo Fisher Scientific, Waltham, MA). All cell lines were grown in a humidified incubator with 5% CO_2 at 37°C for at least 7 days before inoculation to mice. All cell lines were routinely tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Analysis of FLT3-Ligand-expanded DC infected with rMVAs

Dendritic cells from FLT3-ligand supplemented bone marrow cultures were generated as described by Minute L., *et al.*,[20] and infected with increasing amounts of rMVA-Control (5PPC), rMVA-CD40L, rMVA-CD137L, or rMVA-Combo (TCID$_{50}$: 0,3; 0,6; 1,25; 2,5; 5). After 18 h, the infected cells were analyzed for the expression of the co-stimulatory receptors by flow cytometry.

Animal handling

Experiments were performed with 6–8-week-old female C57BL/6 mice with body weights between 18 and 20 g purchased from Harlan Laboratories (Barcelona, Spain). Mice were maintained under pathogen-free conditions and were bred in a temperature-controlled animal facility with a 12 h light–dark cycle. The experimental design was approved by the Ethics Committee for Animal Testing of the University of Navarra (033–[20]).

Tumor implantation and treatment

Exponentially growing ID8.OVA-*Vegf*/GFP and MC38 cells were trypsinized and prepared as a single-cell suspension in ice-cold PBS. Animals were injected intraperitoneally with 300 μL of the ID8.OVA-*Vegf*/GFP and MC38 tumor cell suspension (5×10^6 and 5×10^5 cells, respectively). Seven days after tumor cell inoculation, mice were randomized and treated intraperitoneally with 200 μl of 5×10^7 TCID$_{50}$ of the respective MVA recombinant or PBS (control group). A second dose (boost) of treatment was administered 21 days after tumor cell inoculation when indicated. Mice were weighed and closely monitored for ascites development three times a week. Mice were euthanized if their bodyweight reached 28 g or clear signs of pain and distress appeared.

Samples processing

Peritoneal wash, spleens, and omentum were collected after 3 mL of pre-cold PBS (2% FBS) injection in the peritoneal cavity. After a gentle massage of the peritoneum to dislodge any attached cells, a 23 G needle was used to collect all the fluid. Supernatants from peritoneal washes were frozen at −20°C for further analysis.

Spleens were mechanically disaggregated and filtered through a 70 μm cell strainer (Thermo Fisher Scientific, Waltham, MA). Splenocytes and peritoneal cells were depleted of erythrocytes, and single-cell suspensions were kept at 4°C until further analysis by ELISpot or flow cytometry. Omentum was weighted and frozen in RNAlater Stabilization Solution at −80°C until RNA isolation.

ELISpot and ELISA

Specific T-cell responses were assessed *ex vivo* by a mouse IFN-γ Enzyme-linked Immunosorbent Spot (ELISpot) Assay kit (BD-Biosciences). Ninety-six-well Multiscreen IP Plates (Millipore) were coated with 100 μl of assay diluent containing anti–IFN-γ monoclonal antibody and incubated overnight at 4°C. The plates were washed and then blocked with RPMI-1640 medium containing 10% fetal bovine serum (FBS) for 90 min at room temperature. Splenocytes depleted of erythrocytes were added to wells (4 x 10^5 or 2 × 10^5 cells) and stimulated with $OVA_{257-264}$ peptide (1 μg/mL), $p15E_{604-611}$ peptide (10 μg/mL), 1:10 irradiated (20,000 rads) ID8-*Vegf*/GFP or MC38 tumor cells in 200 μl/well. Prior to use, ID8-*Vegf*/GFP tumor cells as a stimulator were treated with 500 IU/mL of IFN-γ for 24 h to increase MHC-I expression. After 24 h of incubation with stimulators, the IFN-producing cells were measured by ELISpot according to manufacturer´s instructions. IFN-γ release levels were measured using BD OptEIA™ Mouse IFN-γ ELISA Set (BD-Biosciences, NJ) following the manufacturer's recommendations after 48 h of antigen incubation.

Flow cytometry

For peripheral blood MHC-I-tetramer stainings, 100–150 μL of mouse peripheral blood samples collected in 25 μL of Heparine 1% (Mayne Pharma, Raleigh, NC) were directly stained with iTAg Tetramer/PE – H-2K^b $OVA_{257-264}$ (MBL, Nagoya, Japan) in the presence of FcR-Block (anti-CD16/32 clone 93 BioLegend, San Diego, CA) following the manufacturer's instructions. For splenocytes and peritoneal cells, single-cell suspensions were first stained with Zombi NIR Fixable viability kit (BioLegend, San Diego, CA) as a live/dead marker and then with iTAg Tetramer/PE – H-2K^b $OVA_{257-264}$ (MBL, Nagoya, Japan) as described above. Once the samples were stained with the MHC-I -tetramer, co-stainings were performed using the following fluorochrome-labeled antibodies (BioLegend, San Diego, CA): anti-CD3-AF647 (17A2), anti-CD8-BV510 (53–6.7), anti-CD4-BV421 (GK1.5), anti-CD19-BV650 (6D5), and anti-CD45.2-FITC or -PrCPC5 (104). For peripheral blood samples, erythrocytes were lysed with FACS™ Lysis solution (BD-Biosciences, NJ). Flow cytometry was performed using the CytoFLEX S cytometer (Beckman Coulter, Brea, CA). Fluorescence minus one (FMO) or biological comparison controls were used for cell analysis. Data analysis was performed using FlowJo software (TreeStar, Ashland, OR).

mRNA isolation and RNA-seq

Total RNA extraction from omentum was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. The concentration and RNA integrity of samples were determined using the Qubit RNA HS (High Sensitivity) Assay kit (Invitrogen, Waltham, MA) and the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA). mRNA samples were sent to Macrogen Inc. for library preparation using TruSeq Stranded Total RNA and sequenced to a depth of 80 M reads/sample using NovaSeq6000 (Illumina, San Diego, CA).

All sample processing and subsequent bioinformatics analysis were performed on a workstation equipped with 16x Intel Xeon W-2245 @ 4.7 GHz and 256 GB of RAM in a Linux system (Ubuntu 20.04). Quality control of all samples was performed with the FastQC tool (v.0.11.9) (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Before alignment, reads with low quality and adapters were removed using Trimmomatic (v.0.39).[21] Alignment was performed with STAR (v.2.7.9a)[22] with mm39 assembly as reference. The matrix of raw counts was obtained with featureCounts (v.2.0.0)[23] and annotated with Gencode version M27. The analysis of differentially expressed genes was carried out in R/Bioconductor (v.4.1.1) following the bioinformatics workflow provided by edgeR.[24] First, genes with less than 5 counts in all the samples (non-expressed genes) were removed from the analysis before normalization. The datasets were normalized using the TMM (trimmed mean of M-values) normalization, then the log_2CPM values were calculated and the normalized expression matrix was used for the statistical analysis. We selected the set of genes differentially expressed for each comparison using the criteria of $p\ value < .05\%$. Gene set enrichment analysis (GSEA) was performed with fgsea (https://doi.org/10.1101/060012) using the M5-GO:BP and C7-IMMNUNESIGDB gene sets from MSigDB v.7.4 database.[25] Before running GSEA, the lists of expressed genes from the DEg analysis were pre-ranked by their t-statistic value without any type of filtering. The output of fgsea was filtered keeping those enriched pathways with $p\ value < .05\%$ and re-ranked by normalized enrichment score (NES). Raw data are publicly available on the GEO database with the GSE identifier (GSE196024).

Statistical analysis

GraphPad Prism V.8.2.1 software (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Data were analyzed by two-way ANOVA followed by Sidak's followed by multiple comparison test. The survival curves of animals treated with different rMVA vectors were plotted according to the Kaplan–Meier method and were compared using *log-rank* test. Statistical significance was considered when $p < .05$.

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