Suppl. Materials and Methods

Generation of MVA-BN recombinants

The generation of MVA recombinants was carried out as described recently [1]. MVA-BN® was developed by Bavarian Nordic and is deposited at the European Collection of Cell Cultures (ECACC) (V00083008). The generation of a recombinant MVA expressing ovalbumin (MVA-OVA) was previously described [1 2]. The gene encoding for the full length murine 4-1BBL protein was codon-optimized, synthetized (Geneart, Life technologies) and cloned into the MVA-OVA genome to generate MVA-OVA-4-1BBL. The gene encoding for the endogenous murine leukemia virus derived envelope glycoprotein (Gp70) was codon optimized, synthetized (Geneart, Life technologies) and cloned into the MVA and MVA-4-1BBL genome, respectively, to generate MVA-Gp70 and MVA-Gp70-4-1BBL. In these constructs, the native gp70 signal peptide was replaced with the murine IgG kappa signal peptide and two mutations (E552R and A558F) located within the putative immunosuppressive domain of the protein were introduced. The gene encoding for Luciferase (Luc) was synthetized (Geneart, Life technologies) and cloned into the MVA genome, to generate MVA-Luc. The gene encoding for human Flt3 Ligand (hFlt3L) was synthetized (Geneart, Life technologies) and cloned into the MVA-OVA genome, to generate MVA-OVA-hFlt3L. All viruses used in animal experiments were purified twice through a sucrose cushion.

Tumor cell injection

Mice were injected subcutaneously in the flank with $5x10^5$ tumor cells. Regarding B16.OVA and B16.F10, prior to injection cells were admixed with 7 mg/ mL Matrigel (Trevigen). For subcutaneous bilateral tumor experiments, $5x10^5$ and $1x10^5$ CT26.WT tumor cells were injected in the right flank and the left flank, respectively. Tumor rechallenge experiments were performed between 3 and 6 months upon clearance of tumors. Subcutaneous rechallenge was carried out at the opposite flank using $5x10^5$ tumor cells. Intravenous rechallenge was performed injecting $2x10^5$ CT26.WT cells IV. Tumor diameter was measured at regular intervals using a caliper twice a week.

In vivo antibody treatment

For $CD8^+$ T cell depletion, tumor-bearing littermates were inoculated i.p. with 200 µg of rat anti-mouse CD8α (clone 2.43, BioXCell) at days -2, 2, 7, 11 and 15 after immunization. For $CD4^+$ T cell and NK cell depletion, tumor-bearing littermates were inoculated i.p. with 200 µg of rat anti-mouse CD4 (clone GK1.5, BioXCell) or200 µg of rat anti-mouse NK1.1 (clone PK136, BioXCell) at days -1, 3, 6 and 10 after immunization, respectively. 200 µg rat IgG2b (clone LTF2, BioXCell) was used as immunoglobulin isotype control for $CD8^+$ T cell and $CD4^+$ T cell depletion. 200 µg rat IgG2a (clone 2A3, BioXCell) was used as immunoglobulin isotype control for NK cell depletion. For induction of hepatotoxicity, naïve mice received 500 µg of anti-4-1BB antibody (clone 3H3, BioXCell) IV twice per week for two weeks. Alanine aminotransferase (ALT) levels were determined in mouse sera by biochemistry analysis in the Department of Veterinary Medicine (LMU).

Analysis of luciferase expression

For *in vivo* and *ex vivo* analyses of luciferase expression, MVA-Luc-injected mice were anesthetized at the indicated time points and injected i.p. with 100 µL of D-Luciferin (50 µg/mL) (Promega). Mice for *ex vivo* analysis were sacrificed 2 minutes after D-Luciferin injection and the organs were quickly excised and placed into 24-well plates. Luciferase activity images of the animals or of the excised organs were acquired for 2 minutes with an IVIS CCD camera system (Xenogen) and analyzed with the Living Image software package (Perkin Elmer).

Infection of bone marrow-derived macrophages (BMDM) and tumor cells

Bone marrow cells obtained from flushed femurs and tibias of C57BL/6J mice were cultured for 8 days in ultra-low attachment plates (Corning) in the presence of 50 ng/mL recombinant human M-CSF (Peprotech). BMDMs and tumor cells were infected for 20 hours at 37°C 5% CO_2 using the

indicated MVA vectors at a 10 $TCID_{50}$ /cell. Cell culture supernatants were collected and kept at -20°C until further analysis. BMDMs and tumor cells were kept on ice for further flow cytometry analysis.

Cell isolation

When indicated, spleens, lymph nodes (LN), tumors and liver were harvested from mice. Tumors and livers were weighed. Tumors, LN and livers were cut and incubated with 0.1 mg/mL collagenase (Worthington)/ 0.0014% DNase (Roche) for 30 minutes at 37°C. Digestion was stopped by adding 10 µL 0.5M EDTA per mL collagenase/DNase. Tumor single-cell suspensions were obtained by mechanically disrupting tumors through a 100-µm cell strainer (Falcon), followed by filtering through a 40-µm cell strainer (Falcon). Spleen, lung and LN single-cell suspensions were prepared by mechanically disrupting tissues through a 70-µm cell strainer (Falcon). Spleen and tumor samples were subjected to red blood cell lysis (Sigma-Aldrich). Liver single cell suspensions were obtained by collagenase/Dnase digestion as described above, followed by mechanically disrupting the tissue through a 40-µm cell strainer (Falcon) and subsequent centrifugation at 850g for 15 minutes in 36% Percoll (GE Healthcare). Cell pellets were subjected to red blood cell lysis (Sigma-Aldrich).

Blood was collected in PBS containing 2% FCS, 0.1% sodium azide and 2.5 U/ mL heparin. Peripheral blood lymphocytes (PBL) were prepared by lysing erythrocytes with red blood cell lysis buffer (Sigma-Aldrich). Mononuclear cells from the abovementioned organs were washed, resuspended in RPMI+2% FCS, counted and kept on ice until further analysis. $1x10^5$ TrueCount counting beads (BD Biosciences) were added to the tumor cell suspensions.

In vitro T cell assay

B16.F10 tumor cells were infected for 20 hours using indicated MVA vectors at a 10 $TCID_{50}$ /cell. Cells were then washed and harvested. $CD8^+$ T cells from spleens and lymph nodes of $CD45.1^+$ OT-I mice

were purified using EasySep kit according to manufacturer's instructions (StemCell Technology). Purity was checked by flow cytometry.

OT-I $CD8^+$ T cells were cultured in T cell medium [RPMI supplemented with 10% FCS, 1% Pen/Strep, 0.35% β-Mercaptoethanol (Sigma-Aldrich)] in 96-well plates with infected B16.F10 tumor cells at a ratio of 5:1. 48 hours later, 50 µL of culture supernatant was collected and kept at -20°C until further analysis. T cell culture medium supplemented with 10 µg/mL Brefeldin A (BFA, Sigma-Aldrich) was added to the cocultures for additional 5 hours. Cell cocultures were then washed and processed for flow cytometry analysis.

Peptide restimulation

When indicated, mononuclear cells were incubated with 5 µg/mL of MHC class I restricted peptides [$OVA_{257–264}$ (SIINFEKL); $p15E_{604-611}$ (KSPWFTTL); $AH1_{6-14}$ (SPSYVYHQF)] for 5–6 h at 37°C 5% CO_2 in T cell medium and 10 µg/mL BFA. Peptides were purchased from GenScript.

Flow cytometry

Mononuclear cell suspensions, BMDMs or tumor cells were stained for 30 minutes at 4°C in the dark using fixable live/dead viability kits prior to staining (Life Technologies). Mononuclear cells were stained using antibodies from BD Biosciences, eBioscience or Biolegend. A complete list of antibodies and clones used is available upon request. When indicated, cell suspensions were stained using a H-$2k^b$ $OVA_{257-264}$- dextramer (Immudex), a H-$2K^b$ $p15E_{604-611}$-pentamer (ProImmune) or a H-$2L^d$ $AH1_{6-14}$ pentamer (ProImmune). For FoxP3 transcription factor and Ki67 staining cells were fixed using FoxP3 Staining Kit (eBioscience). For intracellular cytokine staining, cell suspensions were stained and fixed for intracellular cytokine detection using IC Fixation & Permeabilization Staining kit (eBioscience). All cells were acquired using a digital flow cytometer (LSR II, BD Biosciences) and data were analyzed with FlowJo software version 10.3 (Tree Star).

Tissue processing for cytokine detection

This protocol was adapted from Moynihan *et al* [3]. Briefly, tissue samples containing Lysing Matrix S beads (MP Biomedicals) were supplemented with lysis buffer (5 µL/mg tumor). Lysis buffer is composed of 50mM Tris-HCl (pH 7.5), 150mM NaCl, 10% Glycerol, 1% Tergitol 70% and 10% FCS. Tissue samples were homogenized in a bead shaker at 50 Hz for 2 minutes. Sample supernatants were collected after centrifugation and frozen at -80°C until Multiplex Luminex assay was performed. To detect human Flt3L in organs of tumor-bearing mice, mice were sacrificed at the indicated timepoints. Tumors and lymph nodes were harvested, cut into 2 mm pieces and cultured for 16 hours in T cell medium. Sample supernatants were collected and frozen at -20°C until further analysis.

Cytokine Luminex Assay

Cytokine concentrations in lysates and cell culture supernatants were determined by Multiplex Luminex assays according to manufacturer´s instructions (Thermo Fisher). Analysis was performed using Masterplex 2010 version 2.0.0.77 (Hitachi Solutions, Ltd.).

Enzyme-linked immunosorbent assay (ELISA)

To detect human Flt3L in sample supernatants, human Flt3L DuoSet ELISA (R&D Systems) according to manufacturer´s instructions was employed. To detect HMGB1 from supernatants after infection, ELISA was performed according to manufacturer´s instructions (IBL International). Plates were read at 450nm in a Sunrise FC plate reader (TECAN).

Quantitative real-time PCR for quantification of MVA-specific DNA genome copies

Genomic DNA (gDNA) was isolated from tissues using QIAamp DNA Mini Kit according to manufacturer's instructions (Qiagen) and quantified in a NanoVue spectrophotometer (Biochrom). Briefly, a standard curve starting at $5x10^7$ genome copies (gcs) was prepared using a plasmid

expressing the open reading frame 082L of MVA, target for detection of MVA backbone DNA. Then, quantitative real-time PCR was performed with TaqMan Gene Expression Master Mix (Thermo-Fisher) using specific primers MVA082L sense 5'-acgtttagccgcctttaatagag-3', MVA082L antisense 5'-tggtcagaactatcgtcgttgg-3', and a fluorescein probe 6FAM-aatcccaccgcctttctggatctc-BBQ. Calculations were performed by the 7500 software of the Real-time PCR system (Applied Biosystems). The software determines a threshold cycle (C_T) for every standard dilution, control and replicate, which is inversely proportional to the logarithm of the quantity of gcs of specific DNA. Based on the standard curve, the software determined the respective number of gcs of the target gene by using the C_T value that is measured for each replicate. The quantity (gcs) of a sample is calculated by the average quantity of its duplicate determination.

1. Medina-Echeverz J, Hinterberger M, Testori M, et al. Synergistic cancer immunotherapy combines MVA-CD40L induced innate and adaptive immunity with tumor targeting antibodies. *Nat Commun* 2019;10(1):5041. doi: 10.1038/s41467-019-12998-6 [published Online First: 2019/11/07]
2. Lauterbach H, Patzold J, Kassub R, et al. Genetic Adjuvantation of Recombinant MVA with CD40L Potentiates CD8 T Cell Mediated Immunity. *Front Immunol* 2013;4:251. doi: 10.3389/fimmu.2013.00251 [published Online First: 2013/08/30]
3. Moynihan KD, Opel CF, Szeto GL, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat Med* 2016;22(12):1402-10. doi: 10.1038/nm.4200 [published Online First: 2016/11/01]