Immunotherapy with MVA-BN®-HER2 induces HER-2-specific Th1-immunity and alters the intratumoral balance of effector and regulatory T cells

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Supplementary Materials and Methods:

Recombinant proteins and synthetic peptides. Recombinant human HER-2 ECD-Fc (rhErbB2/FC Chimera, Cat.# 1129-ER-050) chimeric protein was purchased from R&D Systems (Minneapolis, MN). HER-2 ECD overlapping peptide library (OPL): 15-mer peptides with 11-mer overlaps covering the extracellular domain of HER-2 amino acid sequence were synthesized by and purchased from JPT Peptide Technologies, Inc. (Acton, MA). The MHC class I HER-2 peptide TYLPTNASL (HER-2 p63 [1] purchased from Thermo Electron (Ulm, Germany). HER2 protein 104.1 was produced by Pharmexa (now Affitech, Denmark). The protein sequence of 104.1 is similar to the protein expressed by the MVA-BN®-HER2 with the exception that the trans-membrane domain (9 AA) is absent and the protein has a C-terminal His tag.

Animal treatments with MVA-BN®-vectors. For all immunogenicity studies, MVA-BN®-vectors were injected subcutaneously (s.c.) into 2 separate sites. Virus was given at a volume of 200μL for each site. For tumor efficacy studies mice were treated intraperitoneally (i.p) at the indicated $TCID_{50}$ of MVA-BN®, MVA-BN®-HER2 or HER2+CFA on day 4 after challenge. HER2 formulated in CFA was injected i.p. immediately after preparation at 200μL/mouse. Protein/Complete Freund's Adjuvant (CFA) emulsion was prepared using a 5cc luer-lock syringe.

Detection of antibody responses by ELISA. ELISA plates (Thermo Electron, Waltham, MA, Immulon II HB flat-bottom, Cat.# 62402-972) were coated with recombinant human HER-2 ECD-Fc (rhErbB2/FC Chimera, (R&D Systems, Minneapolis, MN; diluted in coating buffer (200mM Na_2CO_3, pH 9.6) at 2μg/mL (50μl/well)) for one hour at room temperature. For the determination of anti-MVA antibody responses, plates were coated with MVA-BN® at 3E7 $TCID_{50}$ in PBS overnight. Plates were then washed blocked for 1 hour with PBS + 0.05% Tween20. Serial dilutions of mouse sera were added in duplicates. Plates were incubated for one hour, and thoroughly washed prior to adding 50μL/well of detecting antibody (goat anti-mouse IgG-HRP, Southern Biotech at 1:5000 dilution, Birmingham, AL, Cat.# 1030-05). Bound antibodies were detected by adding 100μL/well of TMB chromogenic substrate. Absorbance at 450nm was measured using a Multiskan plate reader (Thermo Electronics, Waltham, MA). Titers were calculated as the last reciprocal of the highest dilution that resulted in a signal at least two times over background (established with serum from mice treated with TBS).

Determination of Th1/ Th2 ratios. To determine the ratio of IgG2a to IgG1 of anti-HER2 antibody, separate assays were performed using secondary antibodies that react specifically with

mouse IgG2a or IgG1, respectively (Cat.# 1080-05, and #1079-05; Southern Biotech, Birmingham, AL).

HER-2 Staining of CT26-HER-2 cells with immunized sera. CT26-HER-2 cells were harvested, washed in PBS, and incubated with sera from treated mice diluted 1:100 in PBS for 2 hours. The anti-HER-2 monoclonal antibody Ab-5 (Calbiochem, San Diego, CA; Cat.# OP39) was used as positive control at a concentration of 0.1mg/mL. Cells were washed and phycoerythrin-conjugated secondary antibody diluted 1:50 in PBS (anti-mouse IgG-PE, Jackson ImmunoResearch, West Grove, PA, Cat.# 115-116-146,) was added for 1 hour. Cells were washed 3 times and analyzed using the BD FACS Calibur and FlowJo software (Tree Star, Inc., Ashland, OR).

Detection of T cell responses by IFN-γ-ELISpot. Membranes of Millipore Multiscreen 96-well filtration plates (Cat.# MAIPS4510) were coated with rat anti-mouse IFN-γ capture antibody (BD Pharmingen, San Diego, CA, Cat.# 551216) at 2μg/mL (50μL/well diluted in PBS) and incubated overnight at 4ºC. Subsequently, plates were washed and blocked with 100μL/well RPMI-10 (RPMI + 10% FCS + β-Mercaptoethanol at $5x10^{-5}$ M) for at least 30 minutes at room temperature. Plates were washed again before splenocytes of treated mice (effector cells) were added in 50μL of RPMI-10 at 5E5 cells per wells. Effector cells were then stimulated by direct addition of Concanavalin A (3.3μg/mL) or serial dilutions of either MVA virus, HER-2 protein, OPL derived from HER-2 ECD, or PSA at concentrations indicated in figure legends. Plates were incubated at 37ºC in a 5% CO_2 incubator. After 40 hours, cells were discarded and plates were washed three times with 150μL/well PBS, and subsequently 3 times with PBS + 0.05% Tween20. Biotin-conjugated monoclonal anti-IFN-γ antibody (Serotech, MCA1548B) was added to all wells at a dilution of 1:5000 in PBS+ 0.05% Tween20 + 5% BSA (50μL/well) and plates were incubated for one to two hours at room temperature. Plates were washed again before Streptavidin-Alkaline Phosphatase (BD Pharmingen, San Diego, CA 554065) at a dilution of 1:5000 in PBS + 0.05% Tween20 + 5% BSA (50μL/well) was added to all wells for 1 hr. Plates were washed and subsequently developed in the dark by adding 50μL/well Vector Blue Substrate (Alkaline Phosphatase Substrate Kit III, Vector Lab Inc., Burlingame, CA, Cat.# SK-5300) for 20 minutes. Plates were scanned and spots counted using an ImmunoSpot plate scanner and ImmunoSpot software version 3.4 (Cellular Technology Ltd., Cleveland OH).

Preparation of cells from tissues for flow cytometry. Single cell suspensions were prepared from lungs after they had been weighed to measure tumor burden. All lungs were pooled together from naïve mice. For tumor challenged groups, the six lungs closest to the value of mean tumor

burden were pooled. Lungs were chopped into fine pieces with a razor blade and incubated in 2.5mL media/lung containing 10% bovine calf serum (BCS), 50 U/mL DNAseI (Spectrum Chemical Mfg. Corp., Gardena, CA) and 250 U/mL Collagenase (Worthington, Lakewood, NJ) for one hour. The dissociated tissue was then squished through a 40 micron cell strainer, cells were pelleted by centrifugation, and red blood cells were lysed with ACK lysis buffer (Lonza, Walkersville, MD). The remaining cells were washed in media, and resuspended in staining buffer containing PBS + 0.1% bovine serum albumin (BSA). In parallel, spleens from the corresponding animals were pooled into RPMI media and single cell suspensions were prepared. Red blood cells were lysed as described for lungs, and splenocytes were resuspended in staining buffer.

Immunophenotyping of lymphocytes by Flow Cytometry. Antibodies against the following proteins were used for staining: CD3e, CD4, CD8a, CD11c, CD19, CD44, CD49b, CD69, CD314 (Beckton Dickinson, Mountain View, CA), and CD25, KLRG-1, and FoxP3 (eBioscience, San Diego, CA). Approximately 50,000 lymphocyte events were acquired per sample using the BD FACS Calibur. Data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). For detection of HER-2-specific $CD8^+$ T cells, H-2 K^d pentamer loaded with the p63 peptide TYLPTNASL was used (ProImmune, Oxford, UK). Approximately 500,000 lymphocyte events were acquired per sample for analysis.

Intracellular IFN-γ Stain (ICS). Splenocytes of treated mice were plated in a sterile V-bottom plate at 1E6 cells/100μL per well. Cells were incubated overnight at 37°C, 5% CO_2 with 100μL per well of a mix of 5μL anti-CD107a Ab (BD Pharmingen, San Diego, CA, Cat. # 553793), 5μL anti-CD107b Ab (BD Pharmingen, San Diego, CA, Cat. # 558758), 0.2μL GolgiStop (BD Pharmingen, San Diego, CA, Cat. # 554724) and the specific antigen for restimulation (p63 peptide), or negative control peptide (PSA overlapping peptide library). Plates were spun down at 1500 RPM for 3 min and washed with 200μL/well PBS with 1% BSA. The pellet was resuspended in 50μL Fc-Block (BD Pharmingen) and incubated for 10 min at RT in the dark. 50μL of anti-CD3 PE (BD Pharmingen, San Diego, CA, Cat. # 553064) at 1:25 and anti-CD8 PerCP-Cy5.5 at 1:50 (BD Pharmingen, San Diego, CA, Cat. # 551162) was added and cells were incubated 20 min at 4°C in the dark. Samples were washed twice with PBS with 1% BSA as above, then 200μL per well of BD Cytofix Buffer (BD Biosciences, San Diego, CA, Cat. # 554714) was added, mixed well, and plates were incubated 20 min at 4°C in the dark. The cells were washed twice with 1X BD Perm/wash Buffer (BD Biosciences, San Diego, CA, Cat. # 554714). Pellets were resuspended in 100μL/well anti-IFN-γ-APC (BD Pharmingen, San Diego, CA, Cat. # 554413) diluted 1:50 in 1X BD Perm/wash Buffer and incubated for 30 minutes at room temperature. Cells

were washed 4 times with 1x BD Perm/Wash Buffer by spinning at 1800 RPM for 3 minutes. Samples were resuspended in 400μL/well of 1% PFA and analyzed using the BD FACS Calibur and FlowJo software (Tree Star, Inc., Ashland, OR).

***In vivo* CTL Assay.** Splenocytes of naïve mice were labeled with indicated peptide concentrations in RPMI-10 (RPMI + 10% FCS + β-Mercaptoethanol (2-ME) at $5x10^{-5}$ M) for 1.5 hours at 37°C, 5% CO_2. Cells were spun down at 1000 RPM for 5 minutes, washed in 50mL PBS and resuspended in 3mL PBS. Then 3mL of either CFSE Stain (Invitrogen, Carlsbad, CA, Cat. # C34554) at 2μM or 0.67μM or eFluor670 dye (eBioscience, San Diego, CA, Cat. # 65-0840) at 5μM, 1.67μM or 0.56μM was added. CFSE stained cells were incubated for 15 minutes at 37°C, 5% CO_2 washed once with RPMI, resuspended in 5mL RPMI-10 and incubated 30 minutes at 37°C, 5% CO_2. Labeled cells were washed twice with 40mL of PBS, and then resuspended in DPBS at a concentration of 3-4 E7 cells per mL. eFluor670 dyed cells were incubated 10 minutes at 37°C, 5% CO_2. Then 30mL RPMI-10 was added and samples were incubated 5 minutes on ice. Cells were washed once with RPMI-10 followed by 2 washes with PBS, and then resuspended in DPBS at a concentration of 3-4 E7 cells per mL. Equal amounts of each labeled cell population was added together and injected in 300μL per mouse via tail vein. The following day the spleens and lungs of the implanted mice were harvested and single cell suspensions were prepared and resuspended in 2mL PBS. Cells were diluted once more 1:4 in PBS and analyzed using the BD FACS Calibur and FlowJo software (Tree Star, Inc., Ashland, OR).

1. Okugawa T, Ikuta Y, Takahashi Y, Obata H, Tanida K, et al. (2000) A novel human HER2-derived peptide homologous to the mouse K(d)-restricted tumor rejection antigen can induce HLA-A24-restricted cytotoxic T lymphocytes in ovarian cancer patients and healthy individuals. Eur J Immunol 30: 3338-3346.