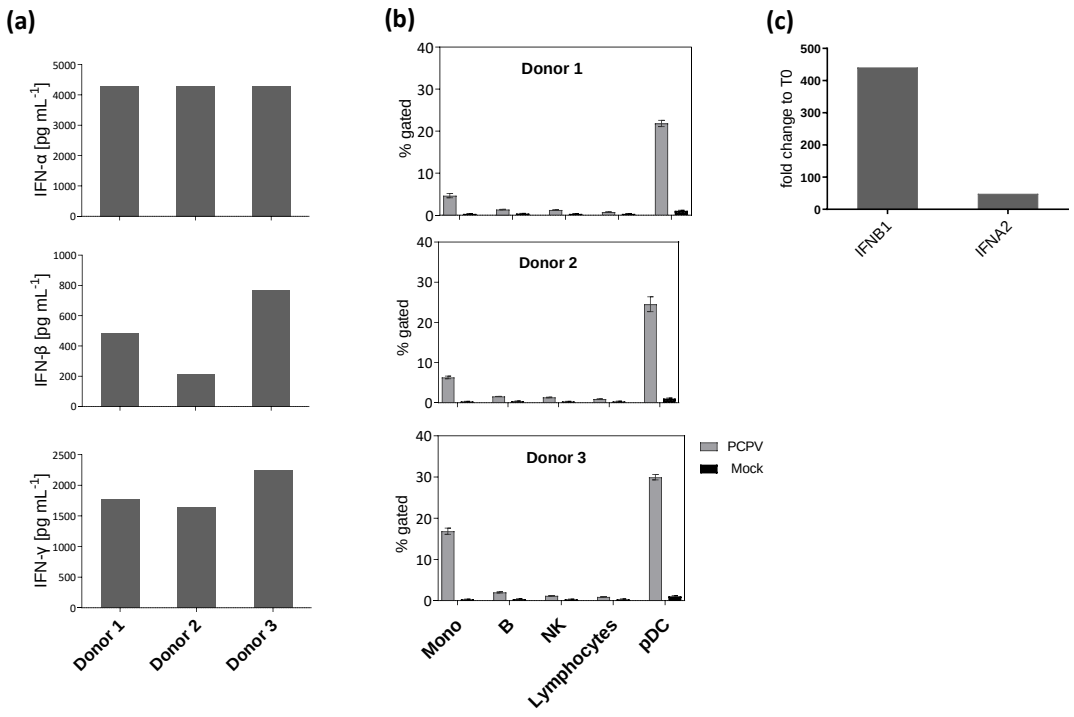
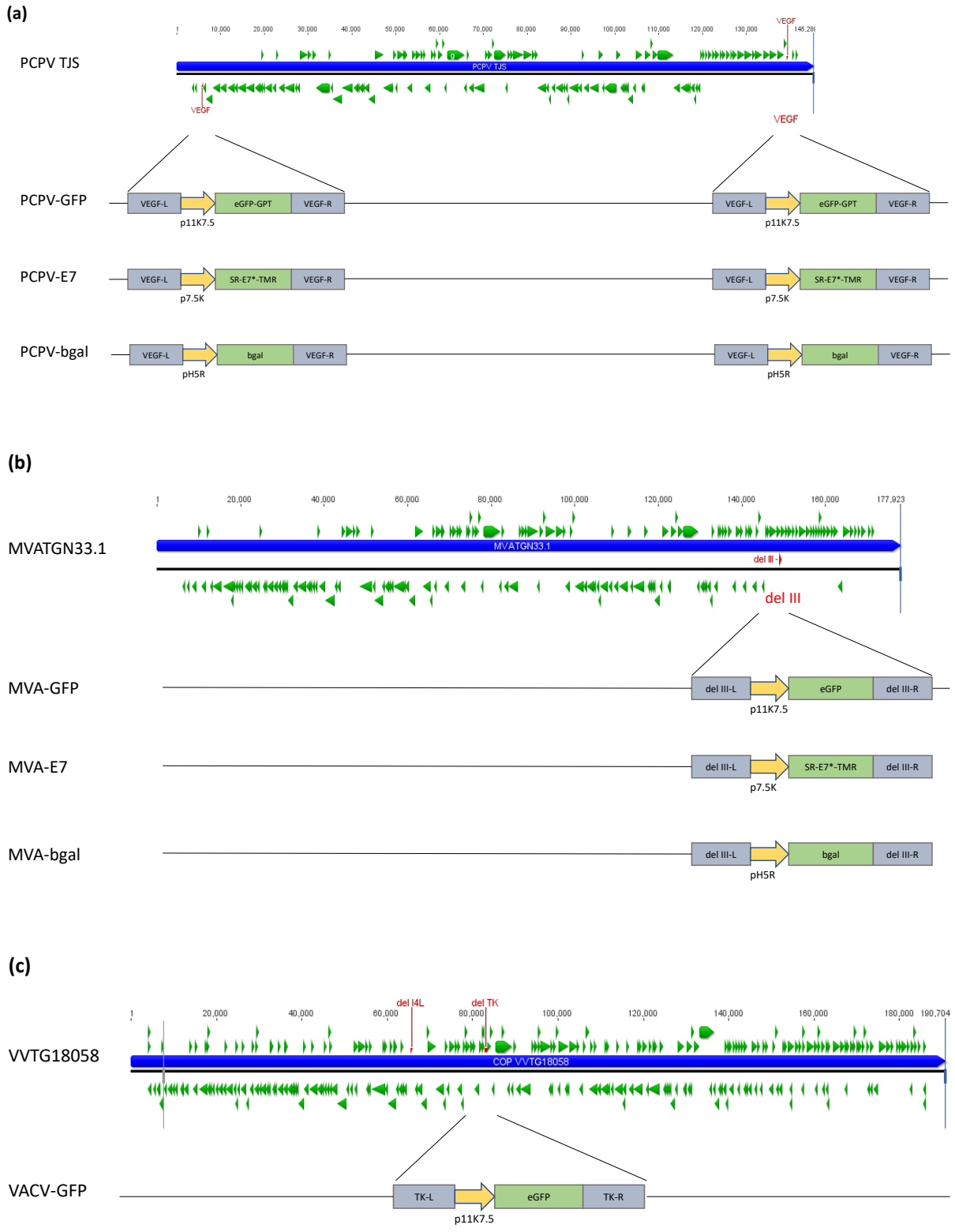
Supplementary figure 1:



Supplementary figure 1. Identification of major IFN-α secreting PBMC subpopulations and secretion of type I and II IFNs. **(a)** PBMCs from three donors were infected with PCPV at MOI 0.3. Dosage of IFN-α, IFN-β and IFN-γ in PCPV infected PBMC supernatants by Luminex analysis. Mean concentrations in mock infected cultures were 1 pg mL^{-1} for IFN-α, 0 pg mL^{-1} for IFN-β and 8 pg mL^{-1} for IFN-γ. **(b)** PBMCs from three donors were infected with PCPV at MOI 0.3 and the IFN-α-secreting cells were identified after o/n incubation by flow cytometry using an antibody capturing IFN-α at the surface of secreting cells (Miltenyi Biotec, Bergisch). PBMC subpopulations were determined with anti-CD3, CD14, CD19, CD16, CD123, CD303. IFN-α-secreting cells were pDCs ($CD303^{+}CD123^{+}$) and monocytes ($CD14^{+}$). **(c)** Transcript analysis in PCPV infected PBMCs: QuantiGene Multiplex Panel (Thermo Fisher Scientific, custom) analysis of mRNA in PCPV-infected PBMCs (MOI 1). Shown is the fold change observed after o/n incubation compared to time-point 0. One of two experiments is shown. The QuantiGenePlex consists of magnetic Luminex xMAP beads ("capture beads") conjugated with

specific capture probe sequences and a target-specific probe set consisting of 3 types of probes: capture extenders, label extenders, and blocking probes. The technical details are described in D. B. Cook, *et al*., Multiplexing protein and gene level measurements on a single Luminex platform. *Methods* **158**, 27-32 (2019). Included gene markers were *IFNB1* and *IFNA2*. The expression levels were standardized against the housekeeping genes *GUSB, POL2RA, HPRT1* and *TBP*.

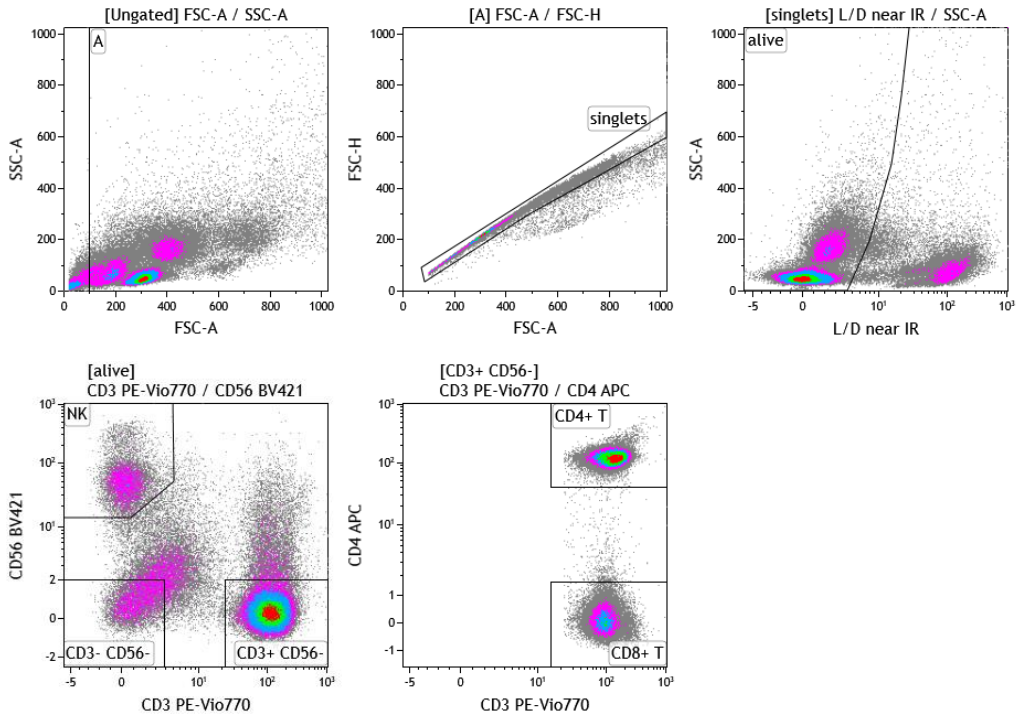
Supplementary figure 2:



Supplementary figure 2. Schematic representation of poxviral recombinant genomes.

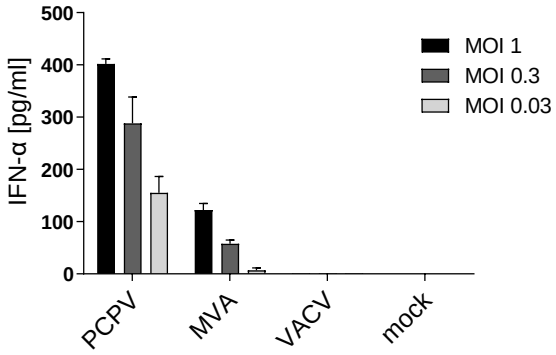
(a) Schematic diagram of the PCPV genome with the two copies of VEGF gene located at each extremity. Both VEGF genes were deleted and were replaced by the transgene sequences. GFP was controlled by the virus-specific promoter p11K7.5 and inserted via homologous recombination between PCPV DNA sequences (VEGL-left and VEGF-right) adjacent to VEGF gene in the PCPV genome. The mutated HPV16 $E7_{(\Delta 21\text{-}26)}$ sequence surrounded by the signal peptide of the glycoprotein precursor of rabies virus ERA strain (SR) and the membrane-anchoring peptide derived from the rabies glycoprotein (TMR) was controlled by the virus specific promoter p7.5K. The beta-galactosidase gene (bgal) from *Escherichia coli* was controlled by the pH5R promoter. These expression cassettes were inserted via homologous recombination as described above. All PCPV viruses were produced for this study. **(b)** Schematic diagram of the MVA genome with the major deletion site III. The site of deletion III served for insertion of the transgene sequences. GFP was controlled by the virus-specific promoter p11K7.5 and inserted via homologous recombination between MVA DNA sequences (del III-left and del III-right) adjacent to deletion site III in the MVA genome. The mutated SR-$E7_{(\Delta 21\text{-}26)}$-TMR sequence was controlled by the virus specific promoter p7.5K. The beta-galactosidase gene (bgal) from *Escherichia coli* was controlled by the pH5R promoter. These expression cassettes were inserted via homologous recombination as described above. All MVA viruses were already produced before this study. **(c)** Schematic diagram of the VACV genome with the deletion in I4L and J2R (TK) genes. The TK gene was deleted and replaced by the transgene sequence. GFP was controlled by the virus-specific promoter p11K7.5 and inserted via homologous recombination between VACV DNA sequences (TK-left and TK-right) adjacent to TK gene in the VACV genome. Both VACV viruses were already produced before this study.

Supplementary figure 3:



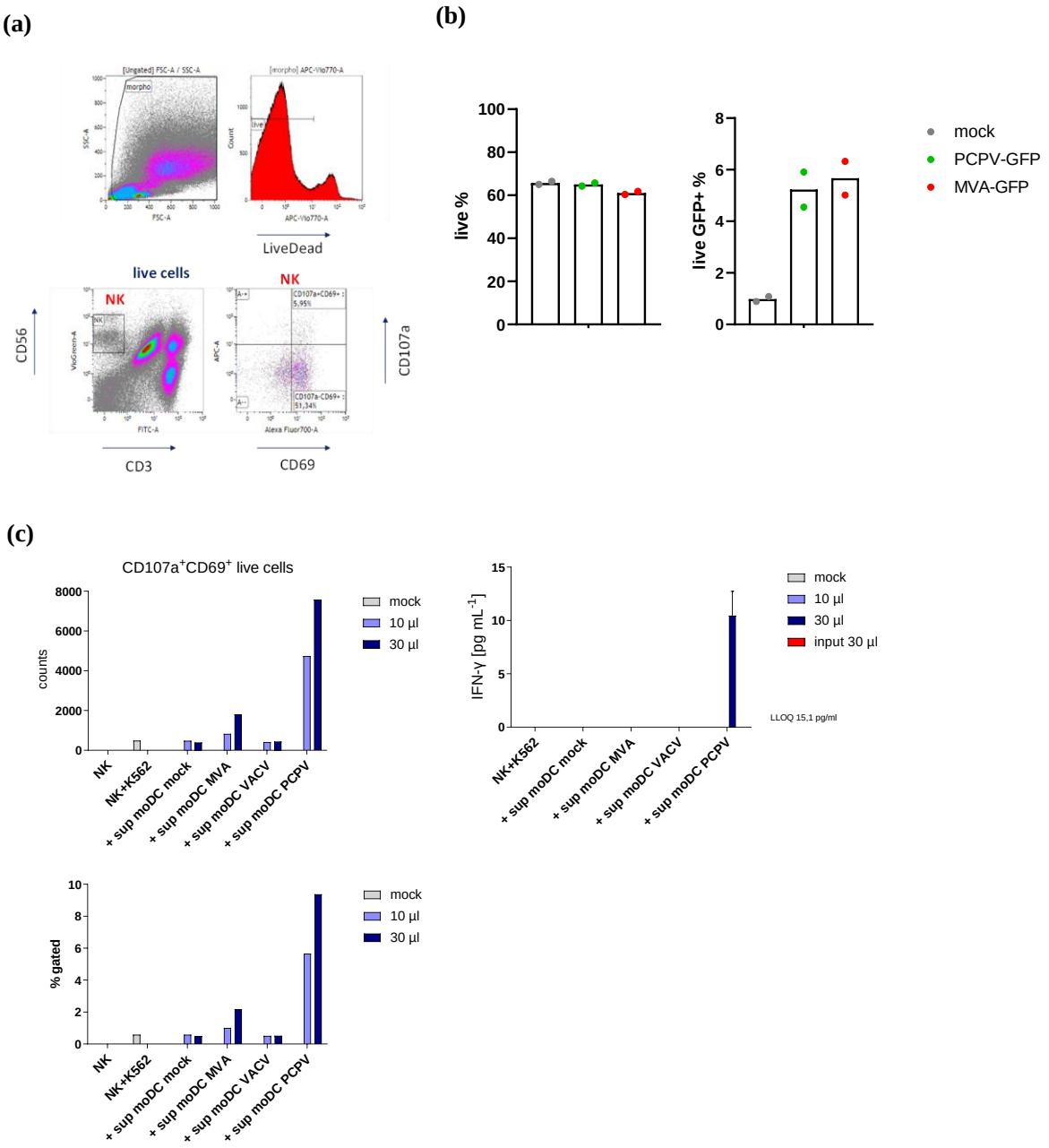
Supplementary figure 3. Representative gating strategy employed for the analysis of PBMC populations infected with GFP-encoding viruses. Shown are the morphology, singlets, live cells, NK population ($CD3^-CD56^+$) within live cells, $CD4^+$ T population within $CD3^+CD56^-$ cells, and $CD4^-$ T population within $CD3^+CD56^-$ cells mainly comprising $CD8^+$ T cells. CD19 was used to identify B lymphocytes within $CD3^-CD56^-$ cells.

Supplementary figure 4:



Supplementary figure 4. MoDC infection with PCPV. MoDCs were infected at the MOIs 0.03, 0.3 and 1 with PCPV, MVA or VACV. IFN-α secreted during o/n incubation into the supernatant was measured. Shown is the mean with SD of triplicates for a single donor. A dose-response relationship could be observed for PCPV and MVA infections. The medium infectious dose of MOI 0.3 was chosen for activation studies with moDCs.

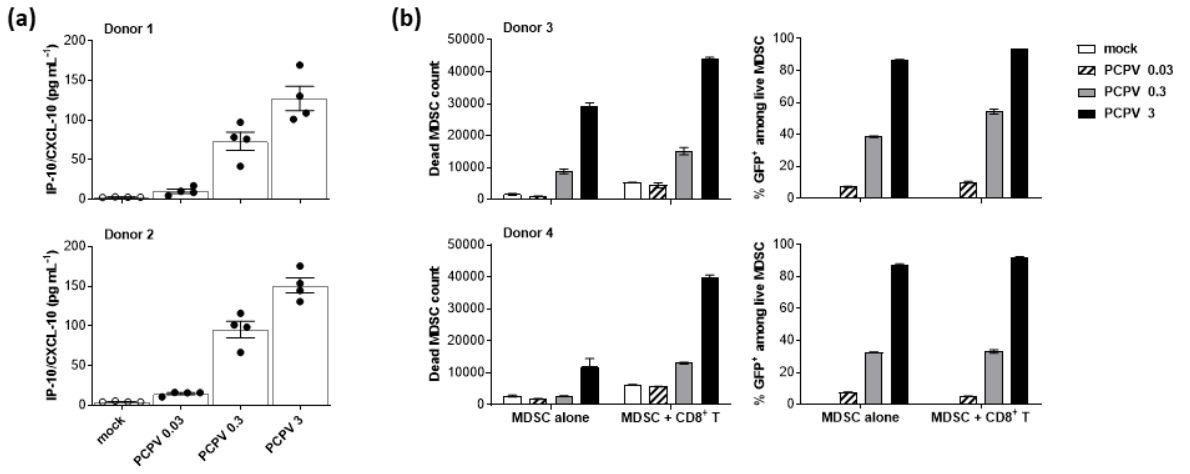
Supplementary figure 5:



Supplementary figure 5. *In vitro* studies on human NK cells. **(a)** Gating strategy to detect degranulating NK cells according to Shabrish *et al.*, 2016. (Morpho →live (near-IR) →$CD3^-$ $CD56^+$ → $CD69^+CD107a^+$). **(b)** Human NK cells were isolated by negative selection from PBMCs from one healthy donor. NK cells were activated by incubation with supernatant from activated moDCs treated with R848 as described in Hart *et al.*, 2005 (doi :

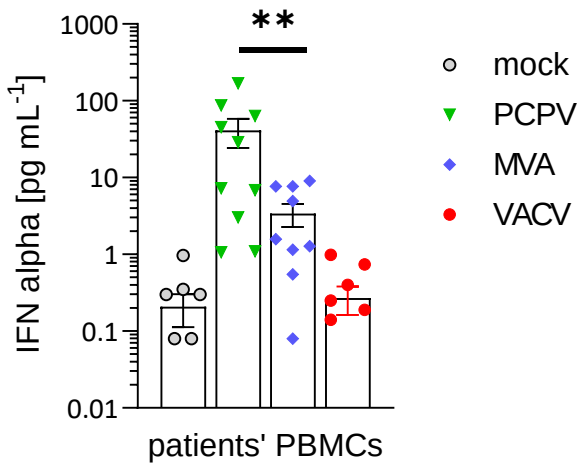
10.4049/jimmunol.175.3.1636). $5x10^5$ activated ($CD69^+$) NK cells were infected with PCPV-GFP and MVA-GFP at an MOI of 1, next day, the frequency of live GFP^+ cells was measured. Infection efficiencies for both viruses were about 5%. Infection did not increase mortality. These data show that activated NK cells are neither efficiently infected nor killed by PCPV (comparable to MVA). **(c)** NK activation is mediated by secreted factors. $5x10^5$ sorted human NK cells, incubated in 500 µl supplemented RPMI, were incubated with 10 or 30 µl of supernatant from $2.5x10^6$ moDCs, infected in 2 ml supplemented RPMI with buffer, MVA, VACV or PCPV at the MOI 0.3. Next day, K562 cells (ratio 1:1) and anti-CD107a were added and degranulation of NK cells was measured one hour later in the $CD69^+$ NK cell population. Best NK degranulation was observed after incubation with supernatant from moDCs infected with PCPV. *De novo* synthesis of IFN-γ was only detected in supernatant from NK cells activated with supernatant from PCPV-infected moDCs.

Supplementary figure 6:



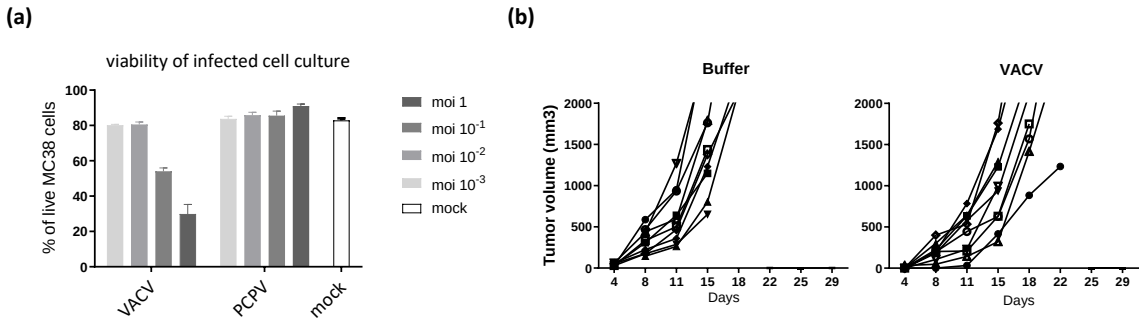
Supplementary figure 6. MDSC studies. **(a)** Concentrations of secreted IP-10 (CXCL-10) in cell culture supernatants following overnight exposure of 1 x 10^5 MDSCs/200 µL to PCPV at MOI 0.03, 0.3 and 3 or in absence (mock) of PCPV. IP-10 production was assessed by Luminex technology. Mean and SEM are indicated. Data are from two independent experiments with one blood donor each. **(b)** Infection with PCPV and subsequent toxicity observed in MDSCs cultured without or with autologous $CD8^+$ T cells (1:1 ratio). Cells were incubated with PCPV-GFP at MOI 0.03, 0.3 and 3, or left untreated (mock) in CELLSTAR® Cell-Repellent Surface 24-well plates (Greiner Bio-One) in presence of T Cell TransAct (Miltenyi Biotec) diluted 1/100 and 20 U mL^{-1} human IL-2. The next day, cells were treated with FcR Blocking Reagent and stained with CD11b-BV421 (ICRF44; BioLegend) and CD5-APC (UCHT2; BioLegend) antibodies in combination with LIVE/DEAD™ near-IR fluorescent reactive dye. Dead MDSC ($SSC^{hi}CD11b^+LIVE/DEAD^{hi}$) singlet numbers and the frequency of GFP^+ cells within live MDSC ($SSC^{hi}CD11b^+LIVE/DEAD^{low}$) singlets were determined by flow cytometry. Mean and SEM are indicated. Data are from two independent experiments with one blood donor each.

Supplementary figure 7:



Supplementary figure 7. IFN-α secretion by PBMCs from cancer patients. IFN-α secretion in PBMC cultures from 10 cancer patients. The patients, between 31 and 78 years of age, had different indications (breast, lung, bladder, and head and neck cancer) and were untreated when blood was drawn. PBMCs were infected at MOI 0.3 with PCPV-GFP, MVA-GFP or VACV-GFP. IFN-α secretion was measured in the cell culture supernatant the next day. Shown is the mean IFN-α secretion in 10 individuals +/- SEM.

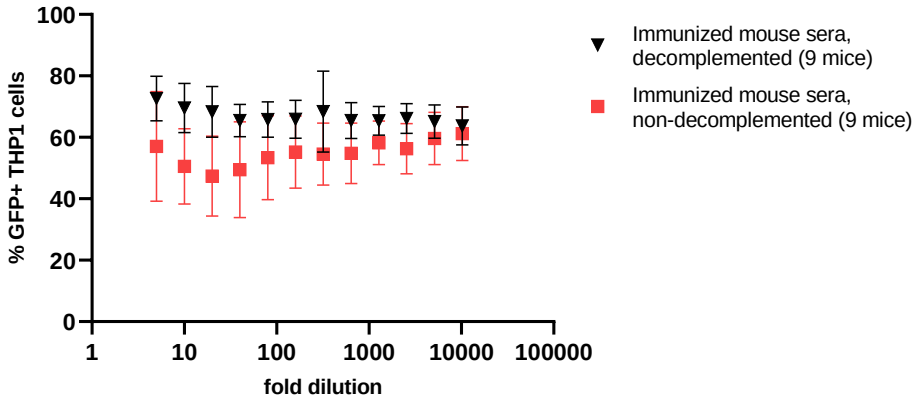
Supplementary figure 8:



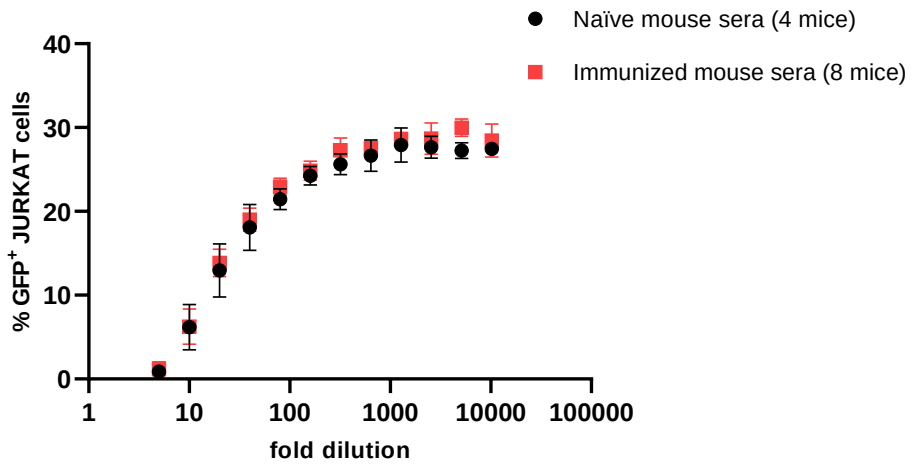
Supplementary figure 8. PCPV infection does not reduce viability of MC38 cells *in vitro*. **(a)** MC38 cells were *in vitro* infected with PCPV or VACV at MOIs ranging from 10^{-3} to 1, or not infected (mock). After 5 days, VACV infection of MC38 cells strongly reduced their viability. MC38 cultures treated with PCPV showed no lytic effects in the infected cells, as evidenced by the same number of live cells at increasing MOIs from 10^{-3} to 1. **(b)** Despite the oncolytic activity of VACV in MC38 *in vitro*, there was no notable tumor control in the MC38 tumor model. A VACV vector was tested according to the protocol described in Figure 3a.

Supplementary figure 9:

(a)



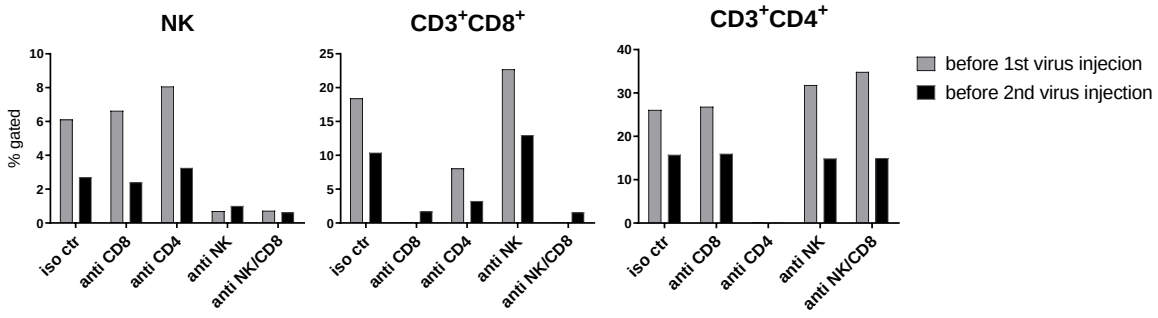
(b)



Supplementary figure 9. Neutralization of infection: C57BL/6 were immunized via the subcutaneous route day 1 and 4, applying $4x10^7$ pfu of a recombinant PCPV vector (PCPV-E7). Day 14, sera from 9 immunized mice (male and female) and naïve mice were harvested. **(a)** PCPV-neutralizing antibodies were not detected: Sera were in part incubated at 56°C for 30

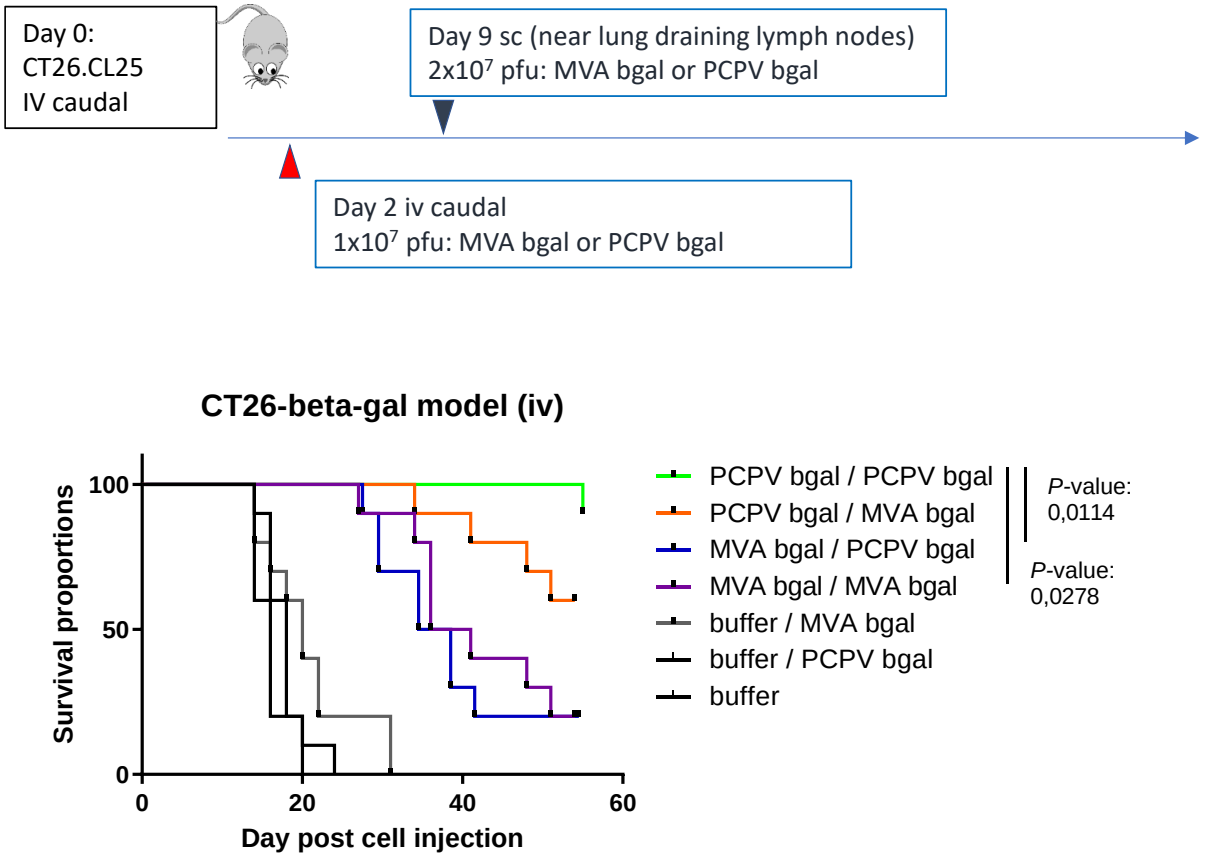
min. The human monocytic cell line THP1 (ATCC, TIB-202) was infected with PCPV-GFP at the MOI of 1, in the presence of the serially diluted sera. The day after infection, the percentage of GFP-positive THP1 cells was determined by flow cytometry. **(b)** Sera from PCPV immunized mice did not reduce infection of Jurkat cells with MVA: The human T cell leukemia cell line Jurkat (ATCC, TIB-152) was infected with MVA-GFP at the MOI 1, in the presence of serially diluted sera from 8 PCPV-immunized mice and 4 naïve mice. The day after infection, the percentage of GFP-positive Jurkat cells was determined by flow cytometry.

Supplementary figure 10:



Supplementary figure 10. Depletion of NK cells and/or $CD8^+$ and $CD4^+$ lymphocytes. Spleens from mice carrying TC1 tumors and treated with antibodies to deplete NK cells and/or $CD8^+$ and $CD4^+$ lymphocytes, were harvested before virus treatments. One mouse per group was analyzed. Two-hundred µg of CD4- (clone GK1.5) or CD8-depleting antibodies (clone 53-6.7), or of the anti-CD8 isotype control (clone 2A3) were injected ip at days 12, 13, 20 and 28 after the implantation of TC1 cells. One-hundred and fifty µg of the NK-depleting antibody NK1.1 (clone PK136) were injected ip at days 11, 13, 15, and then twice per week. At days 14 and 21, just before viruses were injected, the depletion of $CD8^+$, $CD4^+$ lymphocytes or NK cells was analyzed in the spleen. The percentages of NK, $CD3^+CD8^+$ and $CD4^+CD8^+$ cells within individual splenocytes were determined. N =1.

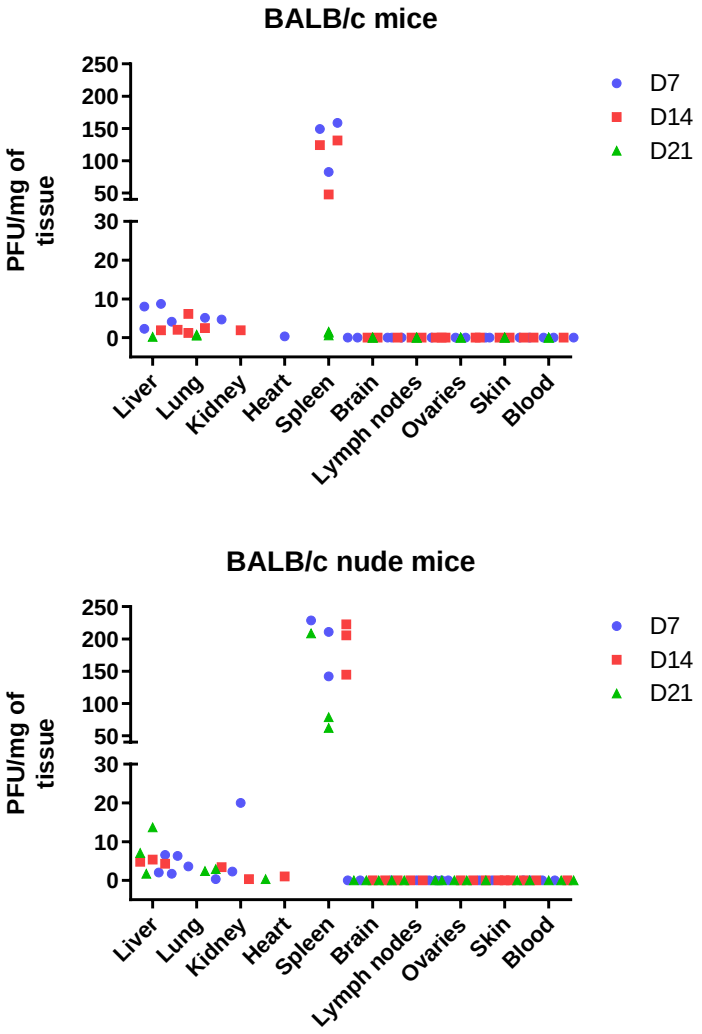
Supplementary figure 11:



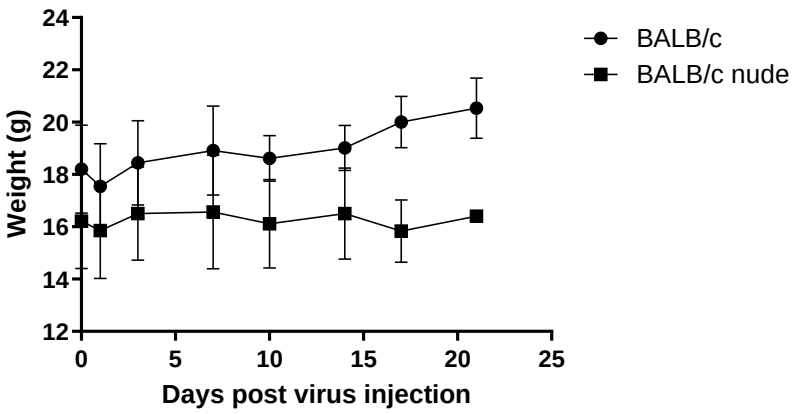
Supplementary figure 11. CT26.CL25 tumor model. Heterologous prime boost with PCPV-bgal/MVA-bgal (described in supplementary figure 2) was comparable to homologous prime boost PCPV-bgal/PCPV-bgal. 10 BALB/c mice per group were iv injected with $2.5x10^5$ CT26.CL25 cells (ATCC CRL-2639). Day 2, the first virus was injected iv at the dose of $1x10^7$ pfu. Since tumors are mainly growing in the lung, the iv route was chosen to assure that incoming virus gets close to the tumor seeds. Day 9, the second virus dose of $2x10^7$ pfu was injected sc in the flank, close to the lung draining lymph nodes. In this model, treatment with PCPV-bgal / PCPV-bgal was well tolerated and resulted with the treatment PCPV-bgal / MVA-bgal in best survival proportions. Groups were compared with a Log-rank test.

Supplementary figure 12:

(a)



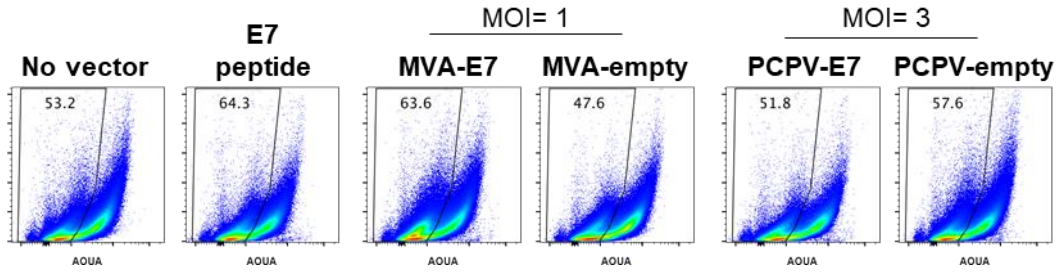
(b)



Supplementary figure 12. Biodistribution of PCPV-GFP in immunocompetent and immunodeficient BALB/c mice. Nine naïve BALB/c and BALB/c J nude mice received iv $1x10^7$ pfu of PCPV encoding GFP. **(a)** Day 7, 14 and 21, three mice were sacrificed, and liver, spleen, brain, lung, kidneys, ovaries, lymphnodes, heart, skin and blood were recovered. Extracts of these organs were used to titrate PCPV on BT cells to detect replication-competent PCPV-GFP, results are expressed as PFU mg^{-1} of tissue. **(b)** Mean weight development of mice during experiment.

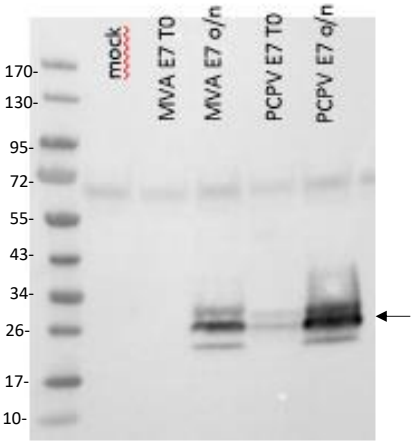
Supplementary figure 13:

(a)



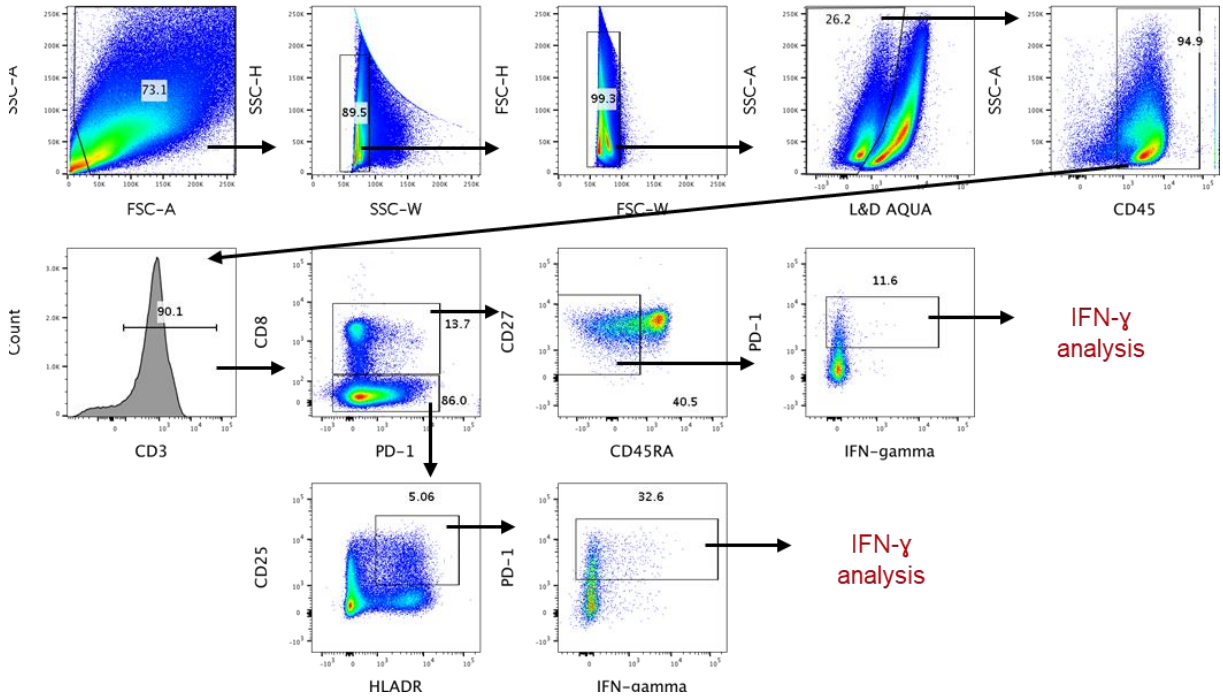
Supplementary figure 13a: Total suspensions of immune cells from head and neck cancer patients' lymph nodes were incubated with distinct MOI of MVA-E7, PCPV-E7 and empty vectors for 10 days. Pseudocolor-plots and graphics shown quantification of live cells by gating on Live&Dead low in distinct conditions.

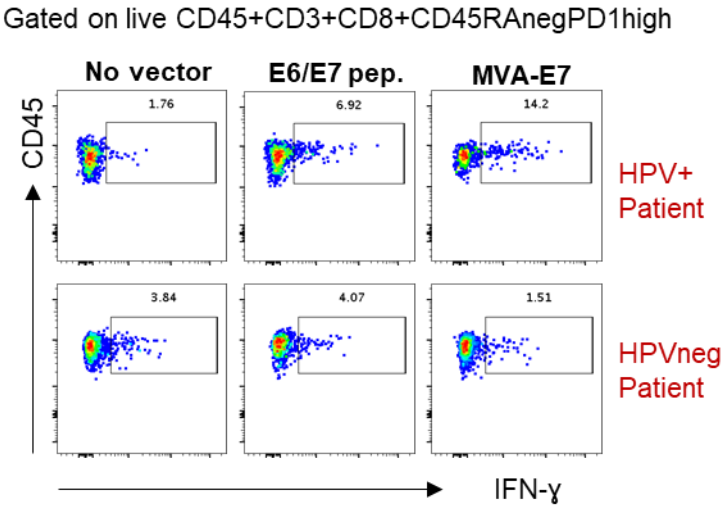
(b)



Supplementary figure 13b: HPV-E7 expression in infected monocytes: $5x10^6$ monocytes were infected at MOI 1 with PCPV-E7 or MVA-E7. The day after, all cells were harvested, samples were run on a Tris-Glycine gel in the presence of 5% beta-mercaptoethanol and transferred for Western blot analysis, using a polyclonal rabbit anti-E7 HPV16 antibody and the ECL Amersham detection system. Molecular weight marker indicated in kDa.

(c)

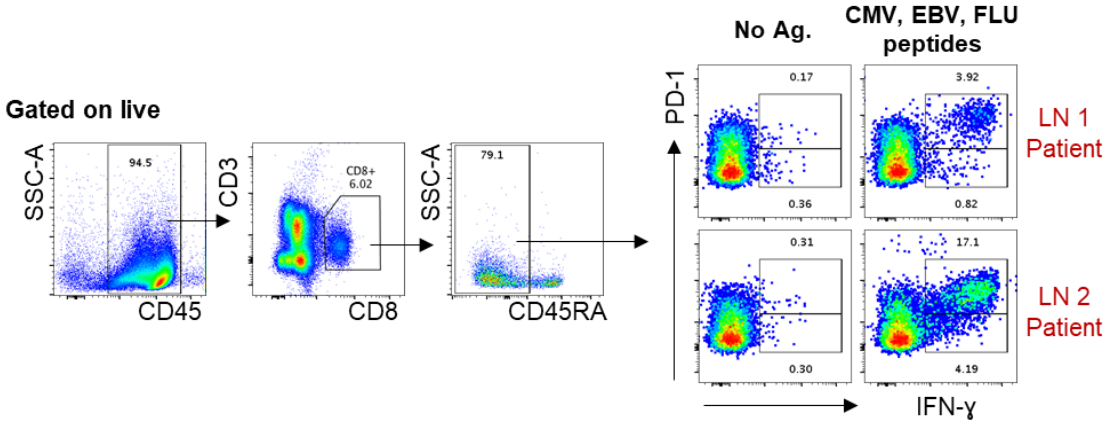




Supplementary figure 13c: Gate strategy and IFN-γ production within activated CD8$^+$ T cells using distinct vectors. Total suspensions of immune cells from head and neck cancer patients' lymph nodes were submitted to expansion protocol during 10 days in the presence of distinct virus vectors. Cells were subsequently re-stimulated by synthetic E7-peptides over-night and IFN-γ production was evaluated by flow cytometry. Upper panel shows gating

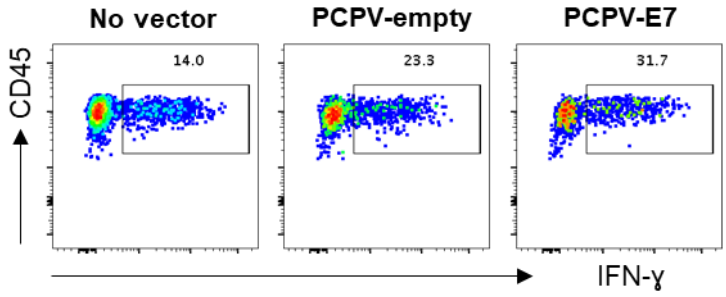
strategy for analysis: For $CD4^+$ T cells, gating strategy used was: live $CD45^+CD3^+CD8^{neg}CD25^+HLADR^+PD1^{high}$ cells; For $CD8^+$ T cells, gating strategy used was: live $CD45^+CD3^+CD8^+CD45RA^{neg}PD1^{high}$ cells. Lower panel shows IFN-γ among memory $CD8^+CD45RA^{neg}PD1^{high}$ T cells after expansion protocol in the presence of synthetic E6/E7 peptides or E7-encoding MVA vector.

(d)



Supplementary figure 13d. Activation of $CD8^+CD45RA^{neg}$ T cells from breast cancer patients' lymph node in the presence of viral antigens. Total suspensions of immune cells from breast cancer patients' lymph nodes were submitted to expansion protocol during 10 days in the presence of CMV, EBV and FLU (CEF) synthetic peptides. Cells were subsequently re-stimulated by synthetic CEF-peptides over-night and IFN-γ production was evaluated by flow cytometry. Representative pseudocolor-plots showing gating strategy and IFN-γ production among live $CD3^+CD8^+CD45RA^{neg}$ cells.

(e)



Supplementary figure 13e: Activation of T cells using empty or E7-encoding PCPV constructs. Total suspensions of immune cells from head and neck cancer patients' lymph nodes were submitted to expansion protocol during 10 days in the presence of PCPV-E7 and PCPV-empty vectors (MOI=3). Cells were subsequently re-stimulated by synthetic E7-peptides over-night and IFN-γ production was evaluated by flow cytometry among live$CD45^{+}CD3^{+}CD8^{neg}HLADR^{+}CD25^{+}PD1^{high}$ activated cells.

Supplementary table 1:

subject ID treatment TG6002 IV (Day 1, 8 and 15)	Study day	PCPV-GFP	MVA-GFP	VVCop-GFP
101010 $3x10^8$ pfu	1	BLT	1/100	1/50
	28	BLT	1/400, 1/800	1/100, 1/200
	43	BLT	1/800, 1/1600	1/200, 1/400
101012 $1x10^9$ pfu	1	BLT	1/50	BLT
	28	BLT	1/800, 1/1600	1/200
	43	BLT	1/800	1/200
702005 $3x10^8$ pfu	1	BLT	1/50, 1/100	BLT
	43	BLT	1/800	1/200, 1/400
702006 $1x10^9$ pfu	1	BLT	BLT	BLT
	28	BLT	1/400, 1/800	1/200, 1/400
	43	BLT	1/200, 1/400	1/100, 1/200

BLT: below limit of titration

Supplementary table 1. PCPV-neutralizing antibodies in serum from cancer patients treated with a VACV vector were not detected. The human monocytic cell line THP-1 (ATCC TIB-202) was infected with GFP-encoding PCPV, MVA or VACV at the MOI of 1. The day after infection, the percentage of GFP-positive THP-1 cells was determined by flow cytometry. To titrate virus-neutralizing antibodies, cells were infected in the presence of serially diluted sera from patients taken before (Day 1) and after treatment (Day 28 and 43) with the VACV-derived virus TG6002 (NCT03724071). The dilution range of the sera needed to obtain 50% neutralization of infection was determined and is shown. BLT (below limit of titration) indicates that even at the lowest dilution, 50% neutralization of infection was not reached.

Supplementary table 2:

Clinical signs	Dose-related piloerection, Dose-related body-weight gain decrease
Biological signs	Slight haematology variations (red blood cells, haematocrit and haemoglobin decrease up to -8%, MCHC decrease up to -4%, RDW, MCV and reticulocytes increases up to +25%, +4% and +70% respectively
Organ weight	Slight spleen weight increase, slight thymus weight decrease
Histology	Increase in germinal center formation in spleen, increased cellularity in lymph nodes, consistent with an adaptative immune response to the virus,

MCHC : Mean Cell Haemoglobin Concentration
RDW : Red Cell Distribution Width
MCV : Mean Cell Haemoglobin

Supplementary table 2. Dose tolerability study in C57BL/6 mice, conducted according to Good Laboratory Practices (GLP). PCPV-E7 was administered iv on Day 1, 4 and 8, at 0 (vehicle), $5x10^5$, $5x10^6$ and $1x10^7$ pfu. Furthermore, the $5x10^6$ pfu intermediate dose was also tested in combination with TG4001 (MVA-HPV16E6-E7), administered at $1x10^6$ pfu on Day 14. Animals were observed up to Day 30. The following endpoints/parameters were evaluated: body weight, food and water consumption, clinical observation, haematology, clinical chemistry, organ weights and gross pathology and histopathology examination.

Based on these findings, the Highest Non-Severely Toxic Dose (HNSTD) is $>1x10^7$ pfu.