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(71) Applicant: **TRANSGENE** [FR/FR]; Parc d'innovation, Boulevard Gonthier d'Andernach, 67400 ILLKIRCH-GRAFFENSTADEN (FR).

(72) Inventors: **RITTNER, Karola**; c/o Transgene, Parc d'innovation, Boulevard Gonthier d'Andernach, 67400 ILLKIRCH-GRAFFENSTADEN (FR). **SILVESTRE, Nathalie**; c/o Transgene, Parc d'innovation, Boulevard Gonthier d'Andernach, 67400 ILLKIRCH-GRAFFENSTADEN (FR). **TOSCH, Caroline**; c/o Transgene, Parc d'innovation, Boulevard Gonthier d'Andernach, 67400 ILLKIRCH-GRAFFENSTADEN (FR).

(74) Agent: **REGIMBEAU**; 20, rue de Chazelles, 75847 PARIS CEDEX 17 (FR).

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(54) **Title:** RECOMBINANT PSEUDOCOWPOX VIRUS ENCODING AN INTERLEUKIN-12

(57) **Abstract:** The present invention relates to the field of viral immunotherapy. In particular, the invention concerns a novel recombinant pseudocowpox virus (PCPV) comprising a nucleic acid molecule inserted in its genome and encoding an interleukine-12, composition thereof as well as their therapeutic use for preventive or treating diseases, and, notably, proliferative diseases like cancers. The present invention also provides methods for generating and amplifying such a PCPV and a method for eliciting or stimulating and/or re-orienting an immune response using such a PCPV. In an embodiment, the recombinant PCPV according to the invention further encodes for a heterologous antigenic polypeptide. The invention may be largely used for the therapeutic vaccination.

RECOMBINANT PSEUDOCOWPOX VIRUS ENCODING AN INTERLEUKIN-12

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the field of viral immunotherapy. In particular, the invention concerns a novel recombinant pseudocowpox virus (PCPV) comprising a nucleic acid molecule inserted in its genome and encoding an interleukine-12, composition thereof as well as their therapeutic use for preventive or treating diseases, and, notably, proliferative diseases like cancers. The present invention also provides methods for generating and amplifying such a PCPV and a method for eliciting or stimulating and/or re-orienting an immune response using such a PCPV.

In an embodiment, the recombinant PCPV according to the invention further encodes for a heterologous antigenic polypeptide. The invention may be largely used for the therapeutic vaccination.

BACKGROUND ART

Immunotherapy seeks to boost the host's immune system to help the body to eradicate pathogens and abnormal cells. Widely used in traditional vaccination, immunotherapy is also being actively investigated as a potential modality for treating severe, chronic, or life-threatening diseases in an attempt to stimulate specific and innate immune responses. A vast number of immunotherapeutics have been described in the literature for decades. In particular, several viral and non-viral vectors have now emerged, all of them having relative advantages and limits making them more appropriate to certain indications (see for example Cattaneo and Russell, 2017, PLOS Pathogens doi: 10.1371/journalppat.1006190; Kaufman et al., 2015, Nature Reviews Drug Discovery 14: 642-661; Gomez et al., 2013 expert Rev Vaccines 12(12): 1395-1416). A huge number of immunotherapy platforms are being evaluated in clinical trials and the number of current clinical studies based on poxvirus therapy, whether oncolytic or not, reflects their interesting therapeutic potential. For example, recombinant vaccinia virus (VV)-based vectors are attractive candidates for their excellent safety profile and their capacity to combine robust cellular antigen-specific immune responses with a generalized stimulation of the innate immune system.

TG4001 is an investigational therapeutic vaccine based on a non-propagative, highly attenuated Vaccinia vector, the modified vaccinia virus Ankara (MVA), which is engineered to express HPV16

antigens (E6 & E7) and an adjuvant (IL-2). TG4001 is designed to have a two-pronged antiviral approach: to alert the immune system specifically to cells presenting the HPV16 E6 and E7 antigens, that can be found in HPV16-related tumors, and to further stimulate the infection-clearing activity of the immune system through interleukin 2 (IL-2). TG4001 has been administered to more than 300 individuals, demonstrating good safety and promising efficacy results. Its mechanism of action and good safety profile make TG4001 an excellent candidate for combinations with other therapies in HPV-mediated solid tumors. This therapeutical cancer vaccine is part of a phase Ib/II trial evaluating the combination of TG4001 and avelumab in patients with HPV-16 positive recurrent or metastatic malignancies (ClinicalTrials.gov identifier NCT03260023).

Another MVA-based viral vector is the TG4050 product. This virus-based therapeutic vaccine is an individualized immunotherapy that encodes neoantigens (patient-specific mutations). TG4050 is designed to stimulate the immune system of patients in order to induce a T-cell response that is able to recognize and destroy tumor cells based on their own neoantigens. This individualized immunotherapy is developed and produced for each patient (WO2018/234506). TG4050 entered into a phase 1–2 clinical trial for patients with ovarian or head and neck cancers (ClinicalTrials.gov Identifiers NCT04183166 and NCT0839524 ; Malone et al. , In: Proceedings of the Annual Meeting of the American Association for Cancer Research 2020; 2020 Apr 27-28 and Jun 22-24. Philadelphia (PA): AACR; Cancer Res 2020;80(16 Suppl):Abstract nr 4566 ; Block et al. In: Proceedings of the American Association for Cancer Research Annual Meeting 2022; 2022 Apr 8-13. Philadelphia (PA): AACR; Cancer Res 2022;82(12_Suppl):Abstract nr CT182 ; Delord et al. Journal of Clinical Oncology 2022 40:16_suppl, 2637-2637).

Parapoxviruses (PPVs) represent different candidates that can be used in vector vaccines. Parapoxvirus belongs to the family Poxviridae and the subfamily Chordopoxvirinae. Parapoxviruses are commonly known as causative agents of dermal diseases in ruminants, leading to papular stomatitis and contagious pustular dermatitis, especially in the regions of the lips, nostrils, oral mucosa, and teats. Like other members of the Poxviridae family, parapoxvirus are relatively large and enveloped double-stranded DNA viruses with ovoid geometries that can infect vertebrates including a wide selection of mammals and humans. Parapoxviruses have a unique spiral coat that distinguishes them from other poxviruses. As for poxviruses, viral replication of parapox is cytoplasmic. Entry into the host cell is achieved by attachment of the viral proteins to host glycosaminoglycans (GAGs) that mediates endocytosis of the virus into the host cell. Fusion with the plasma membrane permits to release the core into the host cytoplasm. Early genes are transcribed in the cytoplasm by viral RNA polymerase. Early expression begins at 30 minutes post-infection. Intermediate phase triggers genomic DNA replication at approximately 100 minutes post-

infection. Late genes are then expressed from 140 min to 48 hours post-infection, producing all structural proteins. Assembly of progeny virions starts in cytoplasmic viral factories, producing a spherical immature particle. This virus particle matures into brick-shaped intracellular mature virion (IMV). IMV virion can be released upon cell lysis or can acquire a second double membrane from trans-Golgi and bud as external enveloped virion (EEV) host receptors, which mediates endocytosis. The virus exits the host cell by existing in occlusion bodies after cell death and remains infectious until it finds another host. Replication-competent as well as inactivated Parapoxviruses are known for their immunomodulating properties (Schulze et al., 2009, Vet Microbiol. 137: 260-7). Parapoxvirus ovis (ORFV), the prototype species of the parapoxvirus genus, has been used successfully in veterinary medicine for increasing general resistance in animal chronically persistent viral infections (see e.g. US6,365,393; WO97/32029 and US2003/0013076) as well as in human medicine for treating HIV (W02006/005529) and considered as oncolytic (Rintoul et al., 2012, Mol. Ther.20(6): 1148-57). Various insertion sites were identified within the ORFV genome (WO97/37031). Notably, recombinant ORFV encoding canine distemper virus (CDV) antigen were used as vaccine against CDV (WO2012/01145) and pseudorabies virus in pigs (Rooij et al., 2010, Vaccine 28(7): 1808-13). Zylexis®, formerly known as Baypamune®, which is a preparation of chemically inactivated ORFV derived from strain D1701 is used for the prophylaxis and therapeutic treatment of infectious diseases and for preventing stress- induced diseases in animals. Inactivated ORFV was shown to induce plasmacytoid dendritic cells (pDC) probably through the engagement of a TLR-9 dependent pathway (Von Buttlar et al., 2014, PLOS One 9(8): e106188). More recently, it has been reported potent cytotoxic activities of a chimeric parapoxvirus (referred as CF189) as an oncolytic immunotherapy in triple negative breast cancer (TNBC) tumors (Choi et al. Surgery. 2018 Feb;163(2):336-342). Active replication of the chimeric ORF virus was detected in the tumor tissues 1 week after its injection and natural killer (NK) cell infiltration was observed in the periphery of virus treated tumor tissues. In the treatment of ovarian cancer, results have shown ORF virus has an effective monotherapy in a murine model of advanced-stage epithelial ovarian cancer, and support the translational potential of ORFV as a NK stimulating immunotherapeutic for treatment of such cancer (Van Vloten et al. J Immunother Cancer 2022;10:e004335). It was also shown that ORFV infection enhances CXCL16 secretion and causes oncolysis of lung cancer cells through immunogenic apoptosis (Wang et al. Front Cell Infect Microbiol. 2022 Jul 25;12:910466).

In the field of viral immunotherapy, more specifically in the use of Parapoxvirus as a vector for the delivery of therapeutic gene, it has also been disclosed the use of Pseudocowpox virus (PCPV) as a therapeutic vaccine.

WO2019/170820 discloses a Pseudocowpox virus (PCPV) encoding a heterologous nucleic acid inserted in its genome and its therapeutic use for preventing or treating proliferative diseases like cancers (Ramos et al. Clin Transl Immunology. 2022 May 8;11(5):e1392). Indeed, it has been shown that PCPV-infected peripheral blood mononuclear cells (PBMC) induced the secretion of very high levels of IFN-alpha in a MOI-dependent way (MOI: Multiplicity of infection). Compared to other parapoxvirus, the levels secreted by the PCPV- infected PBMC are well above the moderate secretion levels of IFN-alpha observed with Swine pox virus (SWPV) and Parapoxvirus ovis (ORFV). Compared with MVA, PCPV induced a 1000-fold higher expression of IFN-alpha in human PBMCs whereas SWPV and ORFV displayed a lower 10 to 100-fold induction. Copenhagen Vaccinia Virus and other oncolytic vectors (e.g. raccoonpoxvirus, fowlpoxvirus, Cotia virus, cowpox virus and Myxomavirus) did not raise the IFN alpha level. The results also showed that Parapoxvirus ovis (ORFV) and Yaba-like disease virus (YLDV) were particularly toxic on infected PBMCs: 90% of cells infected at the MOI of 5 died within 16 hours, while at least 50% of living cells were observed for all other viruses, including PCPV. PCPV was also shown to be superior to MVA and Vaccinia Virus to trigger the expression of CD86 in primary human monocyte-derived dendritic cells (moDCs).
As a recombinant vector, assessment of immunogenicity was shown in a model of a recombinant PCPV encoding for the non-oncogenic form of the E7 protein (deleted for amino acids 21-26) of human papillomavirus type 16 (HPV16), thereafter referred as PCPV-E7. Compared to the MVA encoding the same transgene (MVA-E7), PCPV-E7 vaccinated mice showed a significant increase in the number of E7-specific T cells. Thus, a significantly higher percentage of antigen-specific CD8+ T cells was observed in the PCPV-E7 group compared to MVA-E7. PCPV-E7 as a therapeutic vaccine candidate was also challenged in a translational setting in HPV16+ infected HNSCC (head and neck squamous cell carcinoma) cancer patients, with MVA-E7 vaccine as control. In *ex vivo* studies using tumor-draining lymph nodes (TDLN) from those cancer patients, PCPV-E7 and MVA-E7 stimulation resulted in activation/reinvigoration of E7-specific T cells. The higher induction of co-stimulatory molecules in APCs (Antigen-presenting cells), and the lower toxicity towards activated T cells could underlie the higher efficacy of PCPV-E7.

It is known in the art that IFN-α is able to induce rapid differentiation of monocytes into highly activated dendritic cells called IFN-DCs, which are particularly effective in inducing B and T cell immunity (Rizza et al. Autoimmunity. 2010 Apr;43(3):204-9).
Therefore, there is a need for improvement of vaccine performance of the recombinant PCPV as a therapeutical vaccine vector for treating or preventing proliferative diseases such as cancer. It is of importance that such improvement of antitumor immune responses does not put at risk the patient

safety. There is also a need of improvement of the recombinant PCPV encoding a tumor antigen, so as to amplify the specific immune response against said tumor antigen.

SUMMARY OF THE INVENTION

In the context of the present invention, the Inventors designed a novel recombinant pseudocowpox virus (PCPV) comprising inserted in its genome at least one heterologous gene encoding for an interleukin 12 (IL-12), and methods of generating and using such recombinant PCPV.

Unexpectedly, the Inventors have identified that such recombinant PCPV induced both an upregulation of NK cells (natural killer cells) and an upregulation of activated CD8 T cells (CD8+ T cells) when injected to a patient suffering from a cancer, as it will be further described in the present disclosure.

Also, in the context of the present invention, the Inventors designed a novel recombinant PCPV comprising inserted in its genome:

- at least one heterologous gene encoding for an interleukin 12 (IL-12);
- and at least one heterologous gene encoding for an antigen, preferably a tumor antigen, or a viral antigen.

And methods of generating and using such recombinant PCPV.

Unexpectedly, the Inventors have identified that treatment of a tumor with a recombinant PCPV encoding for an IL-12 and a tumor antigen increased survival of the patient and reduced the tumor growth, and an upregulation of antigen-specific CD8+ T cells, compared to a treatment of a tumor with a recombinant PCPV encoding for the same tumor antigen only (to be understood: without encoding for an IL-12).

Importantly and surprisingly, PCPVs described herein are expected to stimulate or improve immune response, especially in cancer immunotherapy.

In a first aspect, the present invention thus relates to a recombinant pseudocowpox virus (PCPV) comprising at least one heterologous nucleic acid inserted in its genome;

wherein one of the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an interleukin 12 (IL-12).

In one embodiment, said PCPV is obtained from the wild-type TJS strain as identified by ATCC reference number ATCC VR-634™ or from a virus strain of the same or similar name or functional fragments and variants thereof.

In another embodiment, said PCPV is further defective for a viral function encoded by the PCPV genome and preferably is defective for a non-essential viral function and, more preferably for a viral gene function encoded at the insertion site of said heterologous nucleic acid.

In a further embodiment, said heterologous nucleic acid encodes a polypeptide selected from the group consisting of a suicide gene product; an immunomodulatory polypeptide; an antigenic polypeptide; an antibody; a functional derivative of an antibody; a functional fragment of an antibody; and any combination thereof.

Preferably, the immunomodulatory polypeptide is selected from the group consisting of cytokines, such as interleukins, chemokines, interferons, tumor necrosis factor, colony-stimulating factors, polypeptides having an anti-angiogenic effect and agonists or antagonists of immune checkpoints, an any combination thereof.

Also preferred is an immunomodulatory polypeptide that is an interleukin or a colony-stimulating factor and, in particular GM-CSF or is an agonist OX40-directed antibody; or the immunomodulatory polypeptide is a tumor necrosis factor, in particular a CD40L, an IL-4, an IL-21 or a BAFF.

In still a further embodiment, the antigenic polypeptide is a tumor antigen, selected from the group consisting of tumor-associated antigens (TAA), tumor-specific antigens (TSA) and oncoviral antigens. According to an embodiment, said tumor antigen is a class I or class II epitope derived from a tumor antigen, or a combination of class I epitopes, or a combination of class II epitopes, or a combination of class I and class II epitopes.

Preferably, said antigenic polypeptide is a full-length antigen.

In an embodiment, the TAA is selected from the group consisting of WT1, MAGE-A3 MUC1, HER2/Neu and NY-ESO-1; or a tissue specific TAA, preferably a tissue specific TAA selected among Mesothelin, PSA, and gp100.

In another embodiment, the TSA is selected from the group consisting of viral oncogenes; preferably a viral oncogene inducing virus selected among EBV antigens, HPV antigens; or a mutated self TSA, preferably a mutated self TSA selected among EGFRvIII, KRAS, p53 and BRAF; or neo-epitopes resulting from non-synonymous somatic mutations.

In still another embodiment, the oncoviral antigens is selected among viral oncogene inducing virus selected among EBV antigens, HPV antigens.

In still a further embodiment, the at least one heterologous nucleic acid is operably linked to suitable regulatory elements for expression in a desired host cell or subject.

In a preferred embodiment, the at least one heterologous nucleic acid is placed under the control of a poxvirus promoter, preferably, a vaccinia virus promoter and more preferably one selected

from the group consisting of the p7.5K, pH5R, p11K7.5, pSE, pTK, pB2R, p28, p11, pF17R, pA14L, pSE/L, pA35R, pC1R and pK1L promoter, synthetic promoters and early/late chimeric promoters.
In another aspect, the at least one heterologous nucleic acid is inserted in the VEGF locus.

According to an embodiment, the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an interleukin 12 (IL-12), wherein the IL-12 is human IL-12.
In a further embodiment, the IL-12 is a fusion protein comprising an IL-12 p40 subunit and an IL-12 p35 subunit. In a preferred embodiment, the IL-12 p40 subunit is N-terminal to the IL-12 p35 subunit.
In a specific embodiment, the IL-12 p40 subunit comprises the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the amino acid sequence of SEQ ID NO: 1.
In a specific embodiment, the IL-12 p35 subunit comprises the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the amino acid sequence of SEQ ID NO: 2.
According to an embodiment, the IL-12 p40 subunit and the IL-12 p35 subunit are fused in a single polypeptide via an amino acid linker.
Preferably, said amino acid linker is about 5 to about 10 amino acids in length.
More preferably, said amino acid linker is 7 amino acids in length. According to an embodiment, the amino acid linker is a glycine-serine linker. In a specific embodiment, the amino acid linker comprises the amino acid sequence of SEQ ID NO: 3.
According to an embodiment, the IL-12 comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the amino acid sequence of SEQ ID NO: 4. In a preferred embodiment, the IL-12 p40 subunit and the IL-12 p35 subunit are directly fused in a single polypeptide.
According to an embodiment, the nucleic acid sequence encoding the IL-12 comprises a nucleotide sequence at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical to the nucleotide sequence of SEQ ID NO: 5. In an embodiment, the nucleic acid sequence encoding the IL-12 comprises the nucleotide sequence of SEQ ID NO: 5.

In a specific embodiment, the nucleic acid sequence encoding the IL-12 is inserted within the VEGF locus.

In another embodiment, the nucleic acid sequence encoding the IL-12 is inserted within the VEGF locus; and said PCPV further comprises a heterologous nucleic acid encoding for an antigenic polypeptide which is also inserted within the VEGF locus.

In a specific embodiment, the expression cassette comprising the nucleic acid sequence encoding the IL-12 and the expression cassette comprising the nucleic acid encoding the antigenic polypeptide are in a back-to-back orientation in relation to each other within the VEGF locus.

According to an embodiment, the nucleic acid sequence encoding the IL-12 is placed under control of a pSE/L promoter, and the nucleic acid encoding the antigenic polypeptide is placed under control of a p7.5K promoter.

In another aspect, the present invention further provides a method for generating the PCPV of the invention, by homologous recombination between a transfer plasmid comprising the heterologous nucleic acid flanked in 5' and 3' with PCPV sequences respectively present upstream and downstream the insertion site and a PCPV genome, wherein said method comprises a step of generating said transfer plasmid and a step of introducing said transfer plasmid into a suitable host cell, notably together with a PCPV virus comprising the flanking sequence present in the transfer plasmid.

In one embodiment, the site of insertion of the at least one heterologous nucleic acid in the PCPV genome is in a viral gene, with a preference for a non-essential viral gene, in an intergenic region, in a portion of the PCPV genome which does not encode gene products or in a duplicated locus.

In one embodiment, the method for generating the PCPV comprises a step wherein upon insertion of the heterologous nucleic acid in the PCPV genome the viral locus at the insertion site is deleted at least partially, resulting in a defective PCPV virus for said virus function.

According to an aspect, said at least one heterologous nucleic acid is inserted in the VEGF locus.

In a further aspect, the transfer plasmid further comprises one or more selection and/or detectable gene to facilitate identification of the recombinant PCPV.

According to an embodiment, the transfer plasmid is introduced into the host cell in the presence of an endonuclease capable of providing a double-stranded break in said selection or detectable gene.

In one embodiment, the selection gene used in the method for generating the PCPV is the GPT gene encoding a guanine phosphoribosyl transferase permitting growth in a selective medium and/or said detectable gene encodes GFP, e-GFP or mCherry.

According to an embodiment, the suitable host cell is selected from Bovine Turbinate (BT) cell or HeLa cell.

In another aspect, the present invention further provides a method for amplifying the PCPV according to the invention, or generated by the method according to the invention, comprising the steps of a) preparing a producer cell line, b) transfecting or infecting the prepared producer cell line, c) culturing the transfected or infected producer cell line under suitable conditions so as to allow the production of the virus, d) recovering the produced virus from the culture of said producer cell line and optionally e) purifying said recovered virus.

According to an embodiment, the producer cell is Bovine Turbinate (BT) or HeLa.

In another aspect, the present invention further provides a composition comprising a therapeutically effective amount of the PCPV according to the present disclosure, or amplified by the method according to the present disclosure, and a pharmaceutically acceptable vehicle. The composition is thus preferably a pharmaceutical composition.

In an embodiment, said composition is formulated in individual doses comprising from approximately 10^3 to approximately 10^{12} pfu, advantageously from approximately 10^4 pfu to approximately 10^{11} pfu, preferably from approximately 10^5 pfu to approximately 10^{10} pfu; and more preferably from approximately 10^6 pfu to approximately 10^9 pfu of PCPV.

In a further embodiment, the composition is formulated for intravenous, intramuscular, intradermal, intranasal, subcutaneous or intratumoral administration.

In a further aspect, said composition is for use for treating or preventing diseases or pathological condition caused by a pathogenic organism or an unwanted cell division, or for inhibiting tumor cell growth.

It is also disclosed a use of the composition according to the invention for the manufacture of a drug for treating or preventing diseases or pathological condition caused by a pathogenic organism or an unwanted cell division, or for inhibiting tumor cell growth.

It is also disclosed a use of the composition according to the invention for treating or preventing diseases or pathological condition caused by a pathogenic organism or an unwanted cell division, or for inhibiting tumor cell growth.

It is also disclosed a method of treatment comprising administering the composition according to the invention to a subject in need thereof in an amount sufficient to treat or prevent a disease or a pathological condition caused by a pathogenic organism or an unwanted cell division.

It is also disclosed a method for inhibiting tumor cell growth comprising administering the composition of the present invention to a subject in need thereof.

The composition for use, the use or the method according to the present disclosure, comprising 2 to 6 weekly administrations possibly followed by 2 to 15 administrations at 3 weeks interval of the PCPV composition comprising 10^6 to 10^9 pfu.

The composition for use, the use or the method according to the present invention, wherein said method or use is for treating a cancer selected from the group consisting of renal cancer, prostate cancer, breast cancer, bladder cancer, colorectal cancer, lung cancer, liver cancer, gastric cancer, bile duct carcinoma, endometrial cancer, pancreatic cancer, ovarian cancer, head and neck cancer, melanoma, glioblastoma, multiple myeloma, or malignant glioma cells.

The composition for use, the use or the method according to the present invention, which is used in conjunction with one or more other therapeutic agents selected from the group consisting of surgery, radiotherapy, chemotherapy, cryotherapy, hormonal therapy, toxin therapy, immunotherapy, cytokine therapy, targeted cancer therapy, gene therapy, photodynamic therapy and transplantation.

The composition for use, the use or the method according to the present invention, which is carried out according to a prime boost approach which comprises sequential administrations of a priming composition(s) and a boosting composition(s).

The composition for use, the use or the method according to the invention, wherein the priming composition is a PCPV composition, and the boosting composition is a MVA composition or a PCPV composition.

The composition for use, the use or the method according to the present disclosure, whereas the priming composition is a PCPV composition administered by intratumoral route and the boosting composition is a MVA composition administered by intravenous route.

In a further aspect, the composition according to the invention is for use for eliciting or stimulating and/or re-orienting an immune response in a subject in need thereof.

It is also disclosed a use of the composition according to the invention for the manufacture of a drug for eliciting or stimulating and/or re-orienting an immune response in a subject in need thereof.

It is also disclosed a use of the composition according to the invention for eliciting or stimulating and/or re-orienting an immune response in a subject in need thereof.

It is also disclosed a method for eliciting or stimulating and/or re-orienting an immune response comprising administering the composition according to the present invention, to a subject in need thereof, in an amount sufficient to activate the subject's immunity.

Said composition for use, use or method preferably results in at least one the following properties:

- The secretion of high levels of IFN-alpha from PBMC;
- The activation of monocyte-derived dendritic cells;
- The induction of T cell activation or proliferation;
- A better cytokine/chemokine profile in MDSC;
- Activation of APC;
- A M2 to M1 conversion of human macrophages; and/or
- Induction of immunity through a TLR9-mediated pathway or others innate immunity-stimulating pathways.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. IFN-α and IL12-p70 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 and IL12p70 secretion was measured in the cell culture supernatant the next day. Shown is the mean IFN-α and IL12p70 secretion in 10 individuals +/- SEM.

Figure 2. Schematic representation of recombinant PCVPs according to the invention.

Figure 3. A) Western Blot analysis: detection of epitope expression with anti-Flag HRP (horse radish peroxidase) antibody. PCPTG19873 shows around 5-fold higher gene expression than PCPTG19874 B) Quantification of mIL12 in supernatant of infected HeLa cells: PCPV-mIL12 shows 2-fold higher gene expression levels of mIL12 than PCPTG19874.

Figure 4. Murine splenocytes were infected at MOI 0.3 and 1 with PCPV-mIL12 (filled triangle; code number PCPTG19818) or PCPV-ctr (filled quadrangle) or remained uninfected (empty circle). Next day, supernatant was harvested, and IFN-gamma determined by Luminex analysis. Splenocytes were stained with antibodies and Live Dead before being analyzed on a MacsQuant cytometer. Gating strategies using the Kaluza 2.1.1 software (Beckman Coulter): SSC vs FSC. Dot plot (LiveDead vs SSC) to determine live cells. Dot plots of live cells were created to identify the CD3+CD8+,

CD3+CD4+ or the NK cells CD49b+CD3-. From each of these populations, dot plots CD69 vs PDL1 were created to phenotype the activation state of these lymphocytes. Double positive populations were shown. A representative experiment is shown.

Figure 5. PBMCs were infected at MOI 0.3 with PCPV-mIL12 (code number PCPTG19818) or a control PCPV (PCPV-ctr). Next day, supernatant was harvested, and IFN-gamma determined by Luminex analysis. Cells were stained with antibodies and Live Dead before being analyzed on a MacsQuant cytometer. Gating strategies using the Kaluza 2.1.1 software (Beckman Coulter): SSC vs FSC. Dot plot (LiveDead vs SSC) to determine live cells. Dot plots of live cells were created to identify the CD3+CD8+, CD3+CD4+ or the NK cells CD56+CD3-. From each of these populations, dot plots with CD69 vs PDL1 were created to phenotype the activation state of these lymphocytes. Double positive populations were shown. A representative experiment is shown.

Figure 6. PCPV-mIL12 (code number PCPTG19818) controls tumor growth better than PCPV-ctr and increases survival frequencies: Figure 6 A) Two x 10^6 MC38 cells were grafted sc (subcutaneously) in the flank of C57BL/6 mice (10 mice per group). At days 2, 9 and 16, $5x10^6$ pfu of PCPV ctr (dash-dotted line), PCPV-mIL12 (solid line) or buffer (dotted line) were injected into the site of the emerging tumor. Pairwise Log-rank test between all groups: adjusted P-values between buffer and PCPV-mIL-12 (<0,001), PCPV-ctr and PCPV-mIL-12 (0,025). Figure 6 B) Tumor growth in individual mice treated with PCPV-mIL12 shows that 4/10 mice reject tumors (solid lines). In 6/10 mice, tumor growth was controlled up to day 22.

Figure 7. Nine days after MC38 tumor cell grafting, $5x10^5$ pfu of PCPV-ctr, PCPV-mIL12 or the empty MVA vector MVA-ctr were injected into the growing tumors (5 mice per group). The day after, mice were sacrificed, axillary and inguinal draining lymph nodes (DLN) were harvested and analyzed by flow cytometry. Shown are the mean frequencies of activated NK cells, and activated CD8+ and CD4+ T cells in 5 individuals +/- SEM.

Figure 8. Naïve C57BL/6 mice were repeatedly (days 0, 7 and 14) vaccinated (sc) with $1x10^7$ pfu of PCPV-ctr, PCPTG19873or PCPTG19874. Blood was taken at days 7, 14 and 45 (10 mice per group). SIINFEKL-specific T cells were identified by flow cytometry using SIINFEKL-pentamer staining. The day after, mice were sacrificed, axillary and inguinal draining lymph nodes (DLN) were harvested and analyzed by flow cytometry. Shown are the mean frequencies of SIINFEKL-specific CD8+ T cells in 10 individuals +/- SEM.

Figure 9. Comparison of tumor growth and survival frequencies in C57BL/6 mice bearing E.G7-OVA tumors and treated itu (intratumoral) with PCPV-ctr, PCPTG19873 or PCPTG19874 day 5, 12 and 19 after tumor grafting.

Figure 10. Detection of murine IL12 in extracellular vesicle (EV) fraction isolated from infected PBMCs: Suspensions of PCPV-mIL12, incubated o/n with or without PBMCs at MOI 1, were cleared by centrifugation, concentrated, filtered, and ultracentrifuged. mIL12 was quantified after concentration and in EV fraction by Luminex analysis.

Figure 11. Sequence of the expression cassette of the polyepitope inserted within recombinant PCPV referenced as PCPTG19873, and within recombinant PCPV referenced as PCPTG19874. The signs of reference used in Figure 11 are as following:

SP is a signal peptide from the rabies glycoprotein: amino acid 1-23 (P32550.1)

Msln is an epitope from *Mus musculus* Mesothelin: amino acid 396-424 (NP_001343215)

SIINFEKL is a reference for an amino acid sequence known as a pure CD8 epitope

ISQAVHAAHAEINEAGR is a reference for an amino acid sequence known as a pure a pure CD4 epitope

CB100 is an epitope from *Pyrophorus plagiophtalamus* luciferase: amino acid 91-119 (Uniprot S29353)

CB441 is an epitope from *Pyrophorus plagiophtalamus* luciferase: amino acid 430-458 (Uniprot S29353)

Flag is a FLAG tag (Flag HRP (horse radish peroxidase))

Epitopes, signal peptide and flag tag are separated each by a GSG linker

Figure 12. Intracellular cytokine staining (ICS) for IFN gamma in splenocytes taken 45 days after vaccination with PCPV-OVA (PCPTG19873), PCPV-OVA-IL12 (PCPTG19874) or PCPV control (PCPV ctr). Frozen splenocytes were thawed, cultivated, and stimulated for 5 hours in the presence of OVA specific peptide SIINFEKL (OVA) or appropriate controls. Said appropriate controls being DMSO (Dimethyl sulfoxide) or an irrelevant artificial peptide of amino acid sequence KNGENAQAI (referenced as CTRL on Figure 12).

The percentage of live CD8+ T cells secreting IFN-gamma is shown.

Figure 13. Comparison overall survival of PCPV-OVA-IL12 (PCPTG19874), PCPV-OVA (PCPTG19873), MVA-OVA-IL-12 (MVATG20014) and MVA-OVA (MVATG20022) versus their respective control vectors PCPV ctr and MVA ctr in E.G7-OVA tumor model. Log rank test for pairwise comparisons showed significant benefit of survival for PCPTG19874 versus PCPV ctr (adjusted p-value 0.31) and MVATG20014 versus MVA ctr (adjusted p-value (0.002).

Figure 14 (Figure 14A and Figure 14B). Comparison of tumor growth PCPV-OVA-IL12 (PCPTG19874), PCPV-OVA (PCPTG19873), MVA-OVA-IL-12 (MVATG20014) and MVA-OVA (MVATG20022) versus their respective control vectors PCPV ctr and MVA ctr in E.G7-OVA tumor model.

Figure 14A: Measured tumor volume in mm^3

Figure 14B: Graphical representation of an estimated tumor diameter expressed in mm^2 based on mixed model built with measured tumor volume according to Figure 15a and accorded to the method described in Bastogne et al, 2010.

DETAILED DESCRIPTION OF THE INVENTION

In the context of the present invention, the Inventors surprisingly found that a recombinant pseudocowpox virus (PCPV) comprising at least one heterologous nucleic acid inserted in its genome, wherein one of the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an interleukin 12 (IL-12), induced an improve immune response. Indeed, it has been shown that a treatment with such a recombinant PCPV vector induced a dose-dependent increase of the frequency of activated NK cells and CD4+ T cells, and same tendency has been observed for the frequency of activated CD8+ T cells and the secretion of IFN gamma.

It is well known in the art that the presence of NK cells during infection is reported to elicit T-cell exhaustion, and subsequently reduce both CD4 and CD8 T cell response, and that NK cell depletion enhances T-cell mediated viral clearance (Pierce et al. Front Cell Infect Microbiol. 2020 doi: 10.3389).

Therefore, the design of the recombinant PCPV according to the invention having a technical effect to enhance the frequency of activated NK cells, CD4+ T cells and CD8+ T cells is unexpected.

Moreover, in a particular embodiment wherein said PCPV encoding for an interleukin 12 (IL-12) and for a tumor antigen, it has been demonstrated that the survival rate of a treated patient and the reduction of its tumor growth was improved compared to a PCPV encoding for the same tumor antigen alone.

It is an expected improvement, as it is known in the art that IL-12, despite robust antitumor response has not yet been successfully translated into the clinics. Indeed, in clinical trials involving treatment with IL-12, have failed to show sustained antitumor responses, and were associated to toxic side effects (Tugues et al. Cell Death Differ. 2015 doi: 10.1038).

The vectorization of IL-12 into PCPV is therefore a solution to such problem of a vaccination approach for minimizing toxicities and providing antitumor efficacy.

Also, at it will described in the present application, the recombinant PCPV according to the invention showed interesting capacity to produce secreted IL-12 within extracellular vesicles (EVs). It is known in the art that exosome therapeutic that displays functional IL-12 exhibit prolonged tumor retention and greater antitumor activity than a recombinant IL-12 (Lewis et al. Mol Cancer Ther. 2021 Mar;20(3):523-534). Such prolonged retention could increase the effects of IL-12 on tumor-resident NK and T cells, leading to their activation or reinvigoration.

As a consequence, adaptive immune response against the tumor would be strengthened.

General definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

As used herein throughout the entire application, the terms "**a**" and "**an**" are used in the sense that they mean "at least one", "at least a first", "one or more" or "one or a plurality" of the referenced compounds or steps, unless the context dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "**one or more**" refers to either one or a number above one (e.g. 2, 3, 4, etc.).

The term "**and/or**" wherever used herein includes the meaning of "and", "or" and "all or any other combination of the elements connected by said term".

The term "**about**" or "approximately" as used herein means within 10%, preferably within 8%, and more preferably within 5% of a given value or range.

The terms "**amino acids**", "**residues**" and "**amino acid residues**" are used interchangeably and encompass natural amino acids as well as amino acid analogs (e.g. non-natural, synthetic and modified amino acids, including D or L optical isomers).

As used herein, when used to define products, compositions and methods, the term "**comprising**" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are open-

ended and do not exclude additional, unrecited elements or method steps. Thus, a polypeptide "comprises" an amino acid sequence when the amino acid sequence might be part of the final amino acid sequence of the polypeptide. Such a polypeptide can have up to several hundred additional amino acids residues (e.g. linker and targeting peptides as described herein). "**Consisting essentially of**" means excluding other components or steps of any essential significance. Thus, a polypeptide "consists essentially of" an amino acid sequence when such an amino acid sequence is present with eventually only a few additional amino acid residues. "**Consisting of**" means excluding more than trace elements of other components or steps. For example, a polypeptide "consists of" an amino acid sequence when the polypeptide does not contain any amino acids but the recited amino acid sequence.

The terms "**polypeptide**", "**peptide**" and "**protein**" are used interchangeably to refer to polymers of amino acid residues comprising at least nine amino acids covalently linked by peptide bonds. The polymer can be linear, branched or cyclic and may comprise naturally occurring and/or amino acid analogs and it may be interrupted by non-amino acids. No limitation is placed on the maximum number of amino acids comprised in a polypeptide. As a general indication, the term refers to both short polymers (typically designated in the art as peptide) and to longer polymers (typically designated in the art as polypeptide or protein). This term encompasses native polypeptides, modified polypeptides (also designated derivatives, analogs, variants or mutants), polypeptide fragments, polypeptide multimers (e.g. dimers), recombinant polypeptides, fusion polypeptides among others.

Within the context of the present invention, the terms "nucleic acid", "**nucleic acid molecule**", "**polynucleotide**", "**nucleic acid sequence**" and "**nucleotide sequence**" are used interchangeably and define a polymer of at least 9 nucleotide residues in either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or mixed polyribo-polydeoxyribonucleotides. These terms encompass single or double-stranded, linear or circular, natural or synthetic, unmodified or modified versions thereof (e.g. genetically modified polynucleotides; optimized polynucleotides), sense or antisense polynucleotides, chimeric mixture (e.g. RNA-DNA hybrids). Exemplary DNA nucleic acids include without limitation, complementary DNA (cDNA), genomic DNA, plasmid DNA, vectors, viral DNA (e.g. viral genomes, viral vectors), oligonucleotides, probes, primers, coding DNA, non-coding DNA, or any fragment thereof etc. Exemplary RNA nucleic acids include, without limitation, messenger RNA (mRNA), precursor messenger RNA (pre-mRNA), coding RNA, non-coding RNA, etc. Nucleic acid sequences described herein may be synthesized by standard methods known in the art, e.g., by use of an automated DNA synthesizer (such as those that are commercially available from Biosearch, Applied Biosystems, etc.) or obtained from a naturally occurring source (e.g. a genome, cDNA, etc.)

or an artificial source (such as a commercially available library, a plasmid, etc.) using molecular biology techniques well known in the art (e.g. cloning, PCR, etc).

The **percent identities** referred to in the context of the disclosure of the present invention are determined after optimal global alignment of the sequences to be compared, which optimal global alignment may therefore comprise one or more insertions, deletions, truncations and/or substitutions. The alignment is global, meaning that it includes the sequences to be compared taken in their entirety over their entire length. The alignment is "optimal", meaning that the number of insertions, deletions, truncations and/or substitutions is made as low as possible. The optimal global alignment may be performed and the percent identity calculated using any sequence analysis method well-known to the person skilled in the art. In addition to manual comparison, it is possible to determine global alignment using the algorithm of Needleman and Wunsch (1970). For nucleotide sequences, the sequence comparison may be performed using any software well–known to a person skilled in the art, such as the Needle software. The parameters used may notably be the following: "Gap open" equal to 10.0, "Gap extend" equal to 0.5, and the EDNAFULL matrix (NCBI EMBOSS Version NUC4.4). For amino acid sequences, the sequence comparison may be performed using any software well–known to a person skilled in the art, such as the Needle software. The parameters used may notably be the following: "Gap open" equal to 10.0, "Gap extend" equal to 0.5, and the BLOSUM62 matrix.

The terms "**variant**", "**analog**", "**derivative**" and the like can be used interchangeably to refer to a component (polypeptide, nucleic acid, virus, etc) exhibiting one or more modification(s) with respect to the native counterpart. Any modification(s) can be envisaged, including substitution, insertion and/or deletion of one or more nucleotide/amino acid residue(s). Preferred are variants that retain a degree of sequence identity of at least 75%, advantageously at least 80%, desirably at least 85%, preferably at least 90%, more preferably at least 95%, and even more preferably at least 98% identity after optimal global alignment with the sequence of the native counterpart, i.e. after alignment of the sequences to be compared taken in their entirety over their entire length. For illustrative purposes, "at least 75% identity" means 75%, 76%, 77%, 78%, 79%, 80%, 81 %, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%. In a general manner, the term "identity" refers to an amino acid to amino acid or nucleotide to nucleotide correspondence between two polypeptides or nucleic acid sequences. The percentage of identity between two sequences is a function of the number of identical positions shared by the sequences, considering the number of gaps which need to be introduced for optimal alignment and the length of each gap. Various computer programs and mathematical algorithms are available in the art to determine the percentage of identity between amino acid sequences,

such as for example the Blast program available at NCBI or ALIGN in Atlas of Protein Sequence and Structure (Dayhoffed, 1981, Suppl., 3: 482-9). Programs for determining identity between nucleotide sequences are also available in specialized data base (e.g. Genbank, the Wisconsin Sequence Analysis Package, BESTFIT, FASTA and GAP programs). For optimal global alignments, the the algorithm of Needleman and Wunsch (Needleman and Wunsch. J.Mol. Biol. 48,443-453, 1970) may be used, for instance using the Emboss Needle software available at https://www.ebi.ac.uk/Tools/psa/emboss_needle/. This software reads two input sequences and writes their optimal global sequence alignment to file. It uses the Needleman-Wunsch alignment algorithm to find the optimum alignment (including gaps) of two sequences along their entire length. The algorithm uses a dynamic programming method to ensure the alignment is optimum, by exploring all possible alignments and choosing the best. A scoring matrix is read that contains values for every possible residue or nucleotide match. Needle finds the alignment with the maximum possible score where the score of an alignment is equal to the sum of the matches taken from the scoring matrix, minus penalties arising from opening and extending gaps in the aligned sequences. The substitution matrix and gap opening and extension penalties are user-specified. In the context of the invention, in order to obtain an optimal global alignment, the Emboss Needle software may be used with default parameters, i.e. :

- For amino acid sequences: “Gap open” = 10.0,“Gap extend” = 0.5,“End gap penalty” = “false”, “End gap open” = 10.0, “End gap extend” = 0.5, and a “Blosum 62” matrix;
- For nucleotide sequences: “Gap open” = 10.0, “Gap extend” = 0.5, “End gap penalty” = “false”, “End gap open” = 10.0, “End gap extend” = 0.5, and a “DNAfull” matrix.

The term "**obtained from**", “**originating**” or “**originate**” is used to identify the original source of a component (e.g. polypeptide, nucleic acid molecule) but is not meant to limit the method by which the component is made which can be, for example, by chemical synthesis or recombinant means.

As used herein, the term “**host cell**” should be understood broadly without any limitation concerning particular organization in tissue, organ, or isolated cells. Such cells may be of a unique type of cells or a group of different types of cells such as cultured cell lines, primary cells and dividing cells. This term also includes cells that can be or has been the recipient of the non-propagative viral vector for use in the invention, as well as progeny of such cells.

The term “**subject**” generally refers to a vertebrate organism for whom any of the product or methods disclosed herein is needed or may be beneficial. Typically, the organism is a mammal, particularly a mammal selected from the group consisting of domestic animals, farm animals, sport animals, and primates (human and non-human). The terms “subject” and “patient” may be used

interchangeably when referring to a human organism and covers male and female as well as a fetuses, newborn, infant, young adult, adult and elderly.

As used herein, the term "**tumor**" may be used interchangeably with any of the terms "**cancer**", "**malignancy**", "**neoplasm**" and encompasses any disease or pathological condition resulting from uncontrolled cell growth and spread. These terms are meant to include any type of tissue, organ or cell, any stage of malignancy (e.g. from a prelesion to stage IV). Typically, tumors, especially malignant tumors, show partial or complete lack of structural organization and functional coordination as compared to normal tissue and generally show a propensity to invade surrounding tissues (spreading) and/or metastasize to farther sites. The present invention is preferably designed for the treatment of solid tumors as described herein.

A "**neoplastic cell**", "**cancer cell**" or "**tumor cell**" can be used interchangeably to refer to a cell that divides at an abnormal (i.e. increased) rate.

The term "**treatment**" (and any form of treatment such as "treating", "treat", etc.,) as used herein refers to therapy. Typically, therapy refers to a pathological condition with the purpose to improve at least one clinical or biochemical symptom (size of tumor, expression level of associated biomarker…), to slow down or control the progression of the targeted pathological condition, symptom(s) thereof, or a state secondary to the pathological condition in the subject treated in accordance with the present invention.

The terms "**prevention**" (and any form of the term such as "preventing", "prevent", etc.,) and "prophylaxis" are used interchangeably and refer to preventing, delaying the onset or decreasing the severity of the first occurrence or relapse of at least one clinical or biochemical symptom (size of tumor, expression level of associated biomarker, stage progression…).

The term "**administering**" (or any form of administration such as "administered", etc.,) as used herein refers to the delivery to a subject of a component (e.g. the fusion polypeptide according to the invention) according to the modalities described herein.

The term "**combination**" or "**association**" as used herein refers to any arrangement possible of various components (e.g. the fusion polypeptide according to the invention and another treatment). Such an arrangement includes mixture of said components as well as separate combinations for concomitant or sequential administrations. The present invention encompasses combinations comprising equal molar concentrations of each component as well as combinations with very different concentrations. It is appreciated that optimal concentration of each component of the combination can be determined by the artisan skilled in the art.

The terms "**virus**", "virions", "viral particles" and "viral vector particle" are used interchangeably to refer to viral particles that are formed when the nucleic acid vector is transduced into an

appropriate cell or cell line according to suitable conditions allowing the generation of viral particles. In the context of the present invention, the term "viral vector" has to be understood broadly as including nucleic acid vector (e.g. DNA viral vector) as well as viral particles generated thereof. The term "infectious" refers to the ability of a viral vector to infect and enter into a host cell or subject. Viral vectors can be replication-competent or -selective (e.g. engineered to replicate better or selectively in specific host cells), or can be genetically disabled so as to be replication-defective or replication-impaired.

The term "**viral vector**" as used herein refers to a nucleic acid vector that includes at least one element of a virus genome and may be packaged into a viral particle or to a viral particle.

The term "**naturally occurring**", "**native**" or "**wild type**" is used to describe a biological molecule or organism that can be found in nature as distinct from being artificially produced by man. For example, a naturally occurring, native or wild-type virus refers to a virus which can be isolated from a source in nature or obtained from specific collections (e.g. ECCAC, ATCC, CNCM, etc). A biological molecule or an organism which has been intentionally modified by man in the laboratory is not naturally occurring. Representative examples of non- naturally occurring viruses include, among many others, recombinant viruses engineered by insertion of one or more nucleic acid(s) of interest in the viral genome and/or defective virus resulting from one or more modification(s) in the viral genome (e.g. total or partial deletion of a viral gene).

The term "**recombinant**" as used herein in connection with the virus of the present invention indicates that the virus has been modified by the introduction of at least one foreign nucleic acid (also called recombinant gene or nucleic acid, or heterologous gene or heterologous nucleic acid), notably a nucleic acid of therapeutic interest as described herein. In the context of the invention, the "**heterologous nucleic acid**" that is inserted in the PCPV genome is not found in or expressed by a naturally occurring PCPV genome. Nevertheless, the heterologous nucleic acid can be homologous or heterologous to the subject into which the recombinant PCPV is introduced. More specifically, it can be of human origin or not (e.g. of bacterial, yeast or viral origin except PCPV). Advantageously, said heterologous nucleic acid encodes a polypeptide or is a nucleic acid sequence capable of binding at least partially (by hybridization) to a complementary cellular nucleic acid (e.g., DNA, RNA, miRNA) present in a diseased cell with the aim of inhibiting a gene involved in said disease. A polypeptide is understood to be any translational product of a polynucleotide regardless of size, and whether glycosylated or not, and includes peptides and proteins. Such a foreign nucleic acid may be a native gene or portion(s) thereof (e.g. cDNA), or any variant thereof obtained by mutation, deletion, substitution and/or addition of one or more nucleotides.

In a preferred embodiment, the heterologous nucleic acid encodes a polypeptide which is capable of providing a therapeutic or prophylactic activity when administered appropriately to a subject (i.e. a polypeptide of therapeutic interest), leading to a beneficial effect on the course or a symptom of the pathological condition to be treated. A vast number of polypeptides of therapeutic interest may be envisaged. In one embodiment, the heterologous nucleic acid encodes a polypeptide selected from the group consisting of polypeptides that compensate for defective or deficient proteins in a subject, polypeptides that act through toxic effects to limit or remove diseased cells from the body (e.g. suicide gene products); polypeptides capable of potentiating anti-tumor efficacy (e.g. armed gene products); and polypeptides capable of inducing or activating an immune response (such as immunomodulatory and antigenic polypeptides). A heterologous nucleic acid encoding a detectable gene product may also be useful in the context of the invention.

The term, "**immunomodulatory polypeptide**", is used to describe a polypeptide capable of modifying or regulating one or more immune functions (up or downregulation).

The term, "**tumor antigen**", or "**tumoral antigen**", or "**tumoral specific antigen**", is used to describe a protein of peptide that can be recognized by cellular or humoral components of the immune system to target tumor tissue. Tumor is to be understood as abnormal neoplastic tissular formations that might have or not a malignant behaviour. A malignant behaviour is usual associated with spreading and invasion of the nearby tissue and throughout the body. Malignancy is associated with cancer and many non-malignant tumors have the potential to evolve toward a malignant tumor.

The antigen can be a full-length antigen, or an epitope derived from an antigen or an association of epitopes, an epitope being defined as the shortest sequence from an antigen necessary and sufficient to induce a class I or a class II response.

The terms "**tumor-associated antigens**" or "**TAA**"; and "**tumor-specific antigens**" or "**TSA**" are used according to the usual meaning in the art related to the cancer vaccines (see the review article: Lin et al. Nat Cancer 3, 911–926 (2022)).

The term, "**antibody**" ("Ab") is used in the broadest sense and encompasses naturally occurring antibodies and engineered antibodies; including synthetic, monoclonal, polyclonal antibodies as well as full length antibodies and fragments, variants or fusions thereof provided that such fragments, variants or fusions retain binding properties to the target protein. Such antibodies can be of any origin; human or non-human mammal (e.g. rodent or camelid antibody), or chimeric. A nonhuman antibody can be humanized by recombinant methods to reduce its immunogenicity in human. The antibody may derive from any of the well-known isotypes (e.g. IgA, IgG and IgM) and any subclasses of IgG (IgG1, IgG2, IgG3, IgG4). In addition, it may be glycosylated, partially

glycosylated or non-glycosylated. Unless the context indicates otherwise, the term "antibody" also includes an antigen-binding fragment of any of the aforementioned antibodies and includes a monovalent and a divalent fragment and single chain antibodies. The term antibody also includes multi-specific (e.g. bispecific) antibody so long as it exhibits the same binding specificity as the parental antibody. It is within the skill of the artisan to screen for the binding properties of a candidate antibody.

For illustrative purposes, full length antibodies are glycoproteins comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a heavy chain variable region (VH) and a heavy chain constant region which is made of three CH1, CH2 and CH3 domains (optionally with a hinge between CH1 and CH2). Each light chain comprises a light chain variable region (VL) and a light chain constant region which comprises one CL domain. The VH and VL regions comprise three hypervariable regions, named complementarity determining regions (CDR), interspersed with four conserved regions named framework regions (FR) in the following order: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. The CDR regions of the heavy and light chains are determinant for the binding specificity. As used herein, a "humanized antibody" refers to a non-human (e.g. murine, camel, rat, etc) antibody whose protein sequence has been modified to increase its similarity to a human antibody (i.e. produced naturally in humans). The process of humanization is well known in the art and typically is carried out by substituting one or more residue of the FR regions to look like human immunoglobulin sequence whereas the vast majority of the residues of the variable regions (especially the CDRs) are not modified and correspond to those of a non-human immunoglobulin. A "chimeric antibody" comprises one or more element(s) of one species and one or more element(s) of another species, for example, a non-human antibody comprising at least a portion of a constant region (Fc) of a human immunoglobulin.

The antibody is preferably a monoclonal antibody, preferably humanized or chimeric.

Representative examples of antibody fragments and/or regions are known in the art, including heavy (H) chain, light (L), heavy chain variable region (VH), heavy chain constant region, CH domain, light chain variable region (VL), light chain constant region, CL domain, complementarity determining regions (CDR), constant region (Fc), Fab, Fab', F(ab')2, dAb, Fd, Fv, scFv, ds-scFv, diabody, sdAb, etc.

Representative examples of antigen-binding fragments are known in the art, including Fab, Fab', F(ab')2, dAb, Fd, Fv, scFv, ds-scFv, and diabody. A particularly useful antibody fragment is a single chain antibody (scFv) comprising the two domains of a Fv fragment, VL and VH, that are fused together, eventually with a linker to make a single protein chain.

The term "**antigen presenting cell**" (APC), or "**APC**", is used herein as any of various cells (such as dendritic cell, macrophages, or B cell) that uptake and process an antigen in to a peptide fragment which when displayed at the cell surface in combination with a molecule of the major histocompatibility complex is recognized by and serves to activate cells of the immune system (such as helper T cells or cytotoxic T cells). The term "APC-exposed proteins" is used herein as any antigenic protein displayed by an APC on its surface and that may be presented to T-cells in order to trigger an adaptative immune response.

The term "**pseudocowpox virus**" or "**PCPV**" is used herein according to its plain ordinary meaning within Virology and refers to a member of the Poxviridae family which replicates in the cytoplasm of its host and belonging to the Parapoxvirus genus.

Pseudocowpox virus is a virus of the genus Parapoxvirus that causes pseudocowpox in humans and cattle. Pseudocowpox occurs as a common enzootic infection of cattle in most countries of the world. The infection is most frequent in milking herds, affecting the teats and udder of cows and the muzzles and mouths of nursing calves. The lesions of pseudocowpox are characterized by "ring" or "horseshoe"-shaped scabs, the latter being characteristic of the disease. Infection is transmitted by cross-suckling of calves, improperly disinfected teat clusters of milking machines, and probably by the mechanical transfer of virus by flies. Attention to hygiene in the milking shed and the use of teat dips reduce the risk of transmission. Pseudocowpox virus can infect the unprotected hands of people working with affected cattle, causing "milker's nodules" (Fenner's Veterinary Virology (Fifth Edition), 2017).

PCPV possesses a linear and double-stranded DNA genome, typically of 130-150 kilobases. The present invention encompasses naturally occurring forms of pseudocowpox virus of any strain as well as variants thereof which may be modified for various purposes including those described herein.

Representative examples of suitable PCPV strains for use herein include, without limitation, YG2828 (Genbank accession number LC230119), F07.801 R (Genbank accession number JF773693), F10.3081C (Genbank accession number JF773695), F07.798R (Genbank accession number JF773692), F99.177C (Genbank accession number AY453678), IT1303/05 (Genbank accession number JF800906), F00.120R (Genbank accession number GQ329669; Tikkanen et al., 2004, J. Gen. Virol. 85: 1413-8) and TJS (also called VR634; Genbank accession number GQ329670; Friedman-Kien et al., 1963, Science 140: 1335-6; available at ATCC under accession number VR634). Such strains may have morphological, structural and/or genetic differences each other, e.g., in terms of

ITR length, number of predicted genes and/or G C rich content (see e.g. Hautaniemi et al., 2010, J. Gen. Virol.91 : 1560-76).

In a preferred embodiment, the PCPV virus of the present invention is obtained from the wild-type TJS strain as identified by ATCC reference number ATCC VR-634™ or from a virus strain of the same or similar name and functional fragments and variants thereof. Preferably, such a variant maintains at least 75% identity at the nucleotide or amino acid level with at least a segment of 10 kilobase (e.g. a continuous sequence of 10kb) in the wild-type TJS pseudocowpox virus genome.

Exemplary modifications that are appropriate in the context of the present invention include without any limitation insertion(s), substitution(s) and/or deletion(s) of one or more nucleotide(s) within the PCPV genome with the goal of modulating (e.g. increase or decrease) expression of one or more viral gene or virus infectivity when compared to the wild-type pseudocowpox virus (e.g. TJS strain) or increasing its therapeutic efficacy (e.g. increase the ability of the PCPV virus to differentially express in diseased cells relative to healthy cells) or insertion of one or more foreign nucleic acid(s) of therapeutic interest or generating a chimeric virus containing PCPV genomic fragment(s) with ones obtained from a different virus origin.

In one and preferred embodiment, the PCPV of the invention comprises inserted in its genome at least one foreign nucleic acid (e.g. resulting in a recombinant PCPV virus), wherein one of the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an interleukin 12 (IL-12).

Alternatively, or in combination, the PCPV virus may be defective for a viral function encoded by the PCPV genome (e.g. a non-essential viral function), preferably for a viral gene function encoded at the insertion site(s) of the foreign nucleic acid.

Recombinant pseudocowpox virus (PCPV) encoding an interleukine-12

The term "recombinant" as used herein in connection with the virus of the present invention indicates that the virus has been modified by the introduction of at least one foreign nucleic acid (also called recombinant gene or nucleic acid), notably a nucleic acid of therapeutic interest as described herein. In the context of the invention, the “foreign nucleic acid” that is inserted in the PCPV genome is not found in or expressed by a naturally-occurring PCPV genome. Nevertheless, the foreign nucleic acid can be homologous or heterologous to the subject into which the recombinant PCPV is introduced. More specifically, it can be of human origin or not (e.g. of bacterial, yeast or viral origin except PCPV). Advantageously, said foreign nucleic acid encodes a polypeptide or is a nucleic acid sequence capable of binding at least partially (by hybridization) to a

complementary cellular nucleic acid (e.g., DNA, RNA, miRNA) present in a diseased cell with the aim of inhibiting a gene involved in said disease. A polypeptide is understood to be any translational product of a polynucleotide regardless of size, and whether glycosylated or not, and includes peptides and proteins. Such a foreign nucleic acid may be a native gene or portion(s) thereof (e.g. cDNA), or any variant thereof obtained by mutation, deletion, substitution and/or addition of one or more nucleotides.

In a preferred embodiment, the foreign nucleic acid encodes a polypeptide which is capable of providing a therapeutic or prophylactic activity when administered appropriately to a subject (i.e. a polypeptide of therapeutic interest), leading to a beneficial effect on the course or a symptom of the pathological condition to be treated. A vast number of polypeptides of therapeutic interest may be envisaged. In one embodiment, the foreign nucleic acid encodes a polypeptide selected from the group consisting of polypeptides that compensate for defective or deficient proteins in a subject, polypeptides that act through toxic effects to limit or remove diseased cells from the body (e.g. suicide gene products); polypeptides capable of potentiating anti-tumor efficacy (e.g. armed gene products); and polypeptides capable of inducing or activating an immune response (such as immunostimulatory and antigenic polypeptides). A foreign nucleic acid encoding a detectable gene product may also be useful in the context of the invention.

The present Inventors have designed novel recombinant pseudocowpox virus (PCPV) comprising a nucleic acid molecule inserted in its genome and encoding an interleukine-12.

Interleukin-12 (IL-12) has been considered as a potential candidate for anti-cancer therapy and has been introduced into viral vectors such as adenoviral vectors for evaluation. IL-12 is a cytokine with immune-modulating and anti-angiogenic functions. IL-12 acts as a key regulator of cell-mediated immune responses through the induction of T helper 1 differentiation, and it induces cellular immunity by promoting IFN-γ production, proliferation, and cytolytic activity of natural killer and T cells. The multi-functionality of IL-12 has led to investigation of this cytokine as an anti-cancer agent (Manetti et al. J Exp Med. 1993 Apr 1;177(4):1199-204).

It is known in the art that IL-12, despite robust antitumor response has not yet been successfully translated into the clinics. Indeed, in clinical trials involving treatment with IL-12, have failed to show sustained antitumor responses, and were associated to toxic side effects (Tugues S et al. Cell Death Differ. 2015 doi: 10.1038 ; Nguyen et al. Front Immunol. 2020 Oct 15;11:575597 ; Leonard et al. Blood. 1997 Oct 1;90(7):2541-8).

The vectorization of IL-12 into PCPV is therefore a solution to such problem of a vaccination approach for minimizing toxicities and providing antitumor efficacy.

In the embodiment as described in the present application, the human IL-12 encoded into the recombinant PCPV is a fusion protein as a single chain IL-12 protein expressed from PCPV in which the full length p40 subunit was fused, via a G6S linker, to the p35 subunit truncated of its leader sequence (Lieschke et al. Nat Biotechnol. 1997 Jan;15(1):35-40). It is to be understood that such embodiment has a purpose of being an illustrative nucleotide sequence encoding an IL-12 and not to be considered as a limiting embodiment of the present invention.

Recombinant pseudocowpox virus (PCPV) encoding an interleukine-12 and a tumor antigen

In one embodiment, the recombinant pseudocowpox virus (PCPV) comprising at least one heterologous nucleic acid inserted in its genome;
wherein one of the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an interleukin 12 (IL-12);
and wherein one of the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an antigenic polypeptide which is a tumor antigen.
Said tumor antigen may be selected from the group consisting of tumor-associated antigens (TAA), tumor-specific antigens (TSA) and oncoviral antigens.

EXAMPLES

Although the present invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the appended claims.

Materials

Virus

PCPTG19873 is a PCPV expressing five epitopes (see **Figure 11**), among them one sequence known to be a pure CD8 OVA epitope of amino acid sequence [SIINFEKL] and CD4 OVA epitope one sequence known to be a pure CD4 epitope of amino acid sequence [ISQAVHAAHAEINEAGR]. Epitopes are separated by GSG linkers, the polyepitopes are preceded by a signal peptide for the

endoplasmic reticulum (ER) and terminated by a subsequent FLAG tag (Flag HRP (horse radish peroxidase)). Said polyepitopes encoded into PCPV is of nucleotide sequence as disclosed as SEQ ID NO: 9. The polyepitopes expressed amino acid sequence is disclosed as SEQ ID NO: 8.

The expression cassette is under control of the poxviral promoter p7.5K (SEQ ID NO: 7) and inserted in the VEGF loci.

PCPTG19818 is a PCPV expressing a murine IL-12 (mIL-12) of nucleotide sequence disclosed as SEQ ID NO: 11 under the control of the pSE/L promoter (SEQ ID NO: 6) and inserted in the VEGF loci. The mIL-12 amino acid sequence as expressed is disclosed as SEQ ID NO: 10.

PCPTG19874 is a PCPV expressing a murine IL-12 (mIL-12) of nucleotide sequence disclosed as SEQ ID NO: 11 under the control of the pSE/L promoter (SEQ ID NO: 6) and inserted in the VEGF loci. PCPTG19874 is also expressing the same five epitopes (polyepitopes) as PCPTG19873. Said polyepitopes encoded into PCPV is of nucleotide sequence as disclosed as SEQ ID NO: 9. The polyepitopes expressed amino acid sequence is disclosed as SEQ ID NO: 8. The mIL-12 expression cassette and the five epitopes (polyepitopes) expression cassette are inserted into the PCPV genome in a back-to-back orientation in relation to each other, each under separate regulatory control (see **Figure 2**). The expression cassette of the polyepitopes is under control of the poxviral promoter p7.5K (SEQ ID NO: 7). The expression cassette of the mIL-12 is under control of the pSE/L promoter (SEQ ID NO: 6).

MVATG20022 is a MVA expressing four epitopes fused to the C-terminal part of the protein coding for the eGFP. Two epitopes were issued from OVA, one sequence known to be pure CD8 OVA epitope of amino acid sequence [SIINFEKL] and one CD4 OVA epitope of amino acid sequence [ISQAVHAAHAEINEAGR] known to be pure CD4 epitope. The two other epitopes are specific for the male antigen H-Y, one is the Dby epitope of amino acid sequence [NAGFNSNRANSSRSS] and the other one is Uty epitope of amino acid sequence [WMHHNMDLI]. eGFP and polyepitopes are separated by a $(G3S)_3$ linker. Said polyepitopes fusion encoded into MVA is of nucleotide sequence as disclosed as SEQ ID NO: 12. The polyepitopes expressed amino acid sequence is disclosed as SEQ ID NO: 13. The expression cassette is under control of the poxviral promoter pH5R (SEQ ID NO: 14) and inserted in the deletion III locus.

MVATG20014 is a MVA expressing a murine IL-12 (mIL-12) of the nucleotide sequence disclosed as SEQ ID NO: 11 under the control of the p11K7.5 promoter (SEQ ID NO: 15) and inserted in the deletion II locus. MVATG20014 is also expressing the same fusion eGFP-polyepitopes as MVATG20022. The expression cassette of the fusion is under the control of the poxviral promoter pH5R (SEQ ID NO: 14) and is inserted in the deletion III locus.

MVA control, or MVA ctr, is a wild type MVA. Said wild type MVA is accessible via culture collections, like ATCC (e.g.: VR-1508™).

PCPV control, or PCPV ctr, is a wild type PCPV (wild-type TJS strain as identified by ATCC reference number ATCC VR-634™).

EXAMPLE 1: IL-12 not induced by Poxviruses in human primary cells

Cytokine secretion profiles, notably of IFN alpha and Interleukin-12 (IL-12), were analysed in the supernatant of Human Peripheral Blood PBMCs cryopreserved from Cancer Donors (purchased from NeoBiotech via CliniSciences), infected with the human Orthopoxviruses VACV, the MVA, or the bovine Parapoxvirus PCPV (MOI of 0.3, incubation period 16 h). Luminex analysis showed that PCPV induced significantly higher secretion of IFN-alpha than MVA, and that VACV had no effect on IFN-alpha secretion. Based on this observation, PCPV was considered a novel vector to enhance the therapeutic efficacy of antitumor vaccination (see Ramos et al. 2022). Concerning IL12p70 secretion after PCPV infection, there was only a weak tendency of higher analyte concentration, but the overall results showed no significant levels of IL12p70 in neither of the cell culture supernatants (**Figure 1**). The absence of a clear IL-12 signals in infected PBMCs suggest that poxviral infection block this signalling pathway (compare effect of R848 in PBMCs).

Cytokine and chemokine profiles in cell culture supernatants were quantified using an appropriate ProcartaPlex immunoassay (Human Inflammation Panel 20-plex, ThermoFisher). The analysis was carried out according to the manufacturer's recommendations using a MagPix device and the ProcartaPlex Analyst 1.0 software.

EXAMPLE 2: Construction of PCPV-mIL12 +/- CD4 and CD8 epitopes

We sought to introduce IL-12 in the PCPV-infection situation by inserting this cytokine into the PCPV backbone. To follow not only innate immune stimulation but also increase of adaptive immunity, in the present embodiment, we added the murine model antigen as described in the part related to **Figure 11** of the present disclosure into the same vector.

Schematic representation of the three different recombinant PCPVs (PCPTG19818; PCPTG19873; PCPTG19874) are shown on **Figure 2**.

We started our studies with a murine IL-12 (mIL12 or mIL-12) which is active in mice and humans. A fusion of subunits p40 and p35 of mIL12 via a linker $(Gly_4Ser)3$, described to have activity like heterodimer p70 (Lieschke *et al.*, 1997, Nature Biotechnology). The nucleic acid sequence encoding for the fusion protein (SEQ ID NO: 11), under control of pSE/L promoter, was inserted by homologous recombination into the VEGF loci of PCPV to generate PCPV-mIL12 (PCPTG19818).

PCPTG19873 encodes five epitopes, among them one sequence known to be a pure CD8 epitope [SIINFEKL] and one sequence known to be a pure CD4 epitope [ISQAVHAAHAEINEAGR]. Epitopes are separated by GSG linkers, the polyepitopes are preceded by a signal peptide for the endoplasmic reticulum (ER) and terminated by a subsequent FLAG tag (Flag HRP (horse radish peroxidase)). The expression cassette is under control of the poxviral promoter p7.5K and inserted in the VEGF loci.

PCPTG19874 contains mIL12 and epitope encoding cassettes (polyepitopes) in a back-to-back orientation in relation to each other within the VEGF locus.

Recombinant PCPVs were generated and produced in bovine Bos Taurus Turbinate (BT) cells (ATCC CRL-1390) and purified as described in Ramos *et al.*, 2022 and WO2019/170820.

EXAMPLE 3: Gene expression of payloads from recombinant PCPVs

To confirm the expression of the payloads encoded by the various recombinant PCPVs, HeLa cells were infected in triplicates at MOI 1 with the indicated virus. After 48 hours, supernatants were harvested for Luminex analysis (murine IL12 simplex assay, ThermoFisher), and cell extracts were prepared for Western Blot analysis (**Figure 3**).

Both, mIL12 and target epitope expressions in HeLa cells were confirmed.

EXAMPLE 4: Effect of PCPV-mIL12 in murine splenocytes

To compare the effect of PCPV-encoded mIL12 (PCPTG19818) in murine cells in vitro, splenocytes were prepared from C57BL/6 mice and infected at MOI 0.3 and 1 in duplicates with a PCPV vaccine encoding for the murine IL-12 (PCPTG19818) or not encoding for IL-12 (PCPV Ctr). The day after infection, the activation status of CD4+ T cells, CD8+ T cells and NK cells was analyzed by flow cytometry. The secretion of murine IFN gamma was quantified by Luminex analysis using a MagPix device (**Figure 4**).

Activated NK, CD4+T and CD8+T cells were detected after infection with both PCPV vectors, which confirms earlier results about PCPV-induced activation of NK and T cells (Ramos et al., 2022). PCPV-mIL12, however, caused an incremental increase of activated NK cells (1,4-fold), of CD8+ T cell (1,8-fold) and of CD4+ T cells (2,5-fold) compared to PCPV ctr. IFN gamma was only detected in supernatant from splenocytes infected with PCPV-mIL12.

EXAMPLE 5: Effect of PCPV-IL12 in human PBMCs

To translate these results in human and confirm the pro-immunogenic effect of PCPV-IL12 in human we exposed human PBMCs to a PCPV encoding for IL12., PBMCs were isolated from leukocyte concentrates by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare) and infected at MOI 0.3 in duplicate, or treated with recombinant mIL12 (PeproTech 210-12) between 5 and 0,5 ng/ml. On the next day, supernatant was harvested, and cells were analyzed by flow cytometry (MACSQuant) to detect activated NK, CD4+T and CD8+ T cells. Live NearIR-, CD56+CD3-, CD3+CD8+, CD3+CD4+ cell populations were probed for activation markers CD69 and PDL1. Human IFN-gamma secreted in the supernatant was measured by Luminex analysis. A representative experiment is shown on **Figure 5**.

Treatment of PBMCs with recombinant mIL-12 showed dose-dependent increase of the frequency of activated NK and CD4+ T cells, same tendency was observed for the frequency of activated CD8+ T cells and the secretion of IFN gamma. Both PCPV vectors increased the frequency of activated NK and CD8+ and CD4+ T cells, as well as the secretion of IFN gamma. PCPV encoding mIL12 (PCPTG19818) caused an incremental increase of NK cells (1,8-fold) and of CD8+ T cell (1,8-fold) activation, and IFN-gamma secretion (3,5-fold) compared to PCPV-ctr. Recombinant mIL-12 showed dose-dependent effects in human cells.

EXAMPLE 6: Comparison of PCPV-mIL12 vs PCPV wild type (PCPV-ctr) *in vivo*

PCPV has shown efficacy in the murine tumor model MC38: PCPV primed specific T cell response without encoding a tumor antigen (Ramos et al, 2022). We used this model at suboptimal conditions to evaluate PCPV-mIL12 (PCPTG19818) vs PCPV-ctr: At day 0, the colon carcinoma cell line MC38 ($2x10^6$ cells), were grafted subcutaneously (sc) in the flank of 10 C57BL/6 mice. On day 2, 9 and 16, viruses were injected at the suboptimal dose of $5x10^6$ pfu into the tumors (**Figure 6**).

PCPV-mIL12 treatment led to significantly higher survival proportions than PCPV-ctr. In the group of PCPV-mIL12 treated animals, 4/10 animals completely rejected the tumor.

EXAMPLE 7: Activation of immune cells in draining lymph nodes day 1 after sc/itu injection

To phenotype the draining lymph nodes (DLN) populations, DLN from tumor bearing mice were collected and scratched through a cell strainer into culture medium. Cells were washed with phosphate-buffered saline (PBS) by centrifugation before the staining with antibodies including CD45 VioGreen (Miltenyi #130-110-665), CD3 PE Cy7 (BD Pharmingen #552774), FVS575V (BD Horizon #565694), CD8 FITC (BD Pharmingen #553030), CD4 APC Vio770 (Miltenyi #130-118-957), NKp46 R718 (BD Pharmingen #751865), PDL1*BV421 (BD Pharmingen #564716) and CD69**APC (Miltenyi #130-115-461). Same panel with corresponding isotypes were used as controls: Rat IgG2a Isotype control BV421 (BD Pharmingen #562965) and Control REA293 APC (Miltenyi #130-113-434) for 30 min at +4°C. Cells were washed with PBS and suspended in 100 µL MACSQuant Running Buffer for flow cytometry analysis. The following strategy of gating was used using the Kaluza 2.1.1 software (Beckman Coulter). Cells were identified by side scatter forward scatter (SSC vs FSC). From this gate, a dot plot (FVS757V vs SSC) was created to determine the Live cells (FVS575V negative). From the live cells, a dot plot CD45 vs SSC was created to gate on CD45+ cells. From this gate, three dot plots were created to identify either the CD3+CD8+, CD3+CD4+ or the NK cells NKp46+CD3-. From each of these populations, dot plots CD69 vs PDL1 were created to phenotype the activation state of these lymphocytes (**Figure 7**).

PCPV vectors increased the frequencies of activated NK and T cells. PCPV-mIL12 (PCPTG19818) further increased these frequencies. MVA-ctr had little effects on NK cells and no effects on T cells.

On murine tumor model, PCPV-mIL12 (PCPTG19818) augments survival and tumor control compared to PCPV-ctr. Cases of complete tumor rejection were observed. The frequency of activated NK, CD8+T and CD4+T cells in DLN Day 1 post-injection was increased.

The frequency of activated NK and CD8+T and CD4+ T cells was higher after injection of PCPV vectors compared to the MVA vector. As the specific response is forged in lymphatic organs such as the draining nodes, an increase in activated immune cells in these organs is a demonstration of a positive effect on the adaptive response.

EXAMPLE 8: Immunogenicity of PCPV-polyepitopes vs PCPV-polyepitopes-mIL12

To assess the immunogenicity, $1x10^7$ pfu of a PCPV-ctr vector, or the polyepitopes encoding vectors PCPTG19873 and PCPTG19874 were injected days 0, 7 and 14 into the flank (subcutaneously) of C57BL/6 mice. At days 7, 14 and 45, blood was taken quantify CD8+ OVA-specific T cells. Spleens were harvested at the end of the experiment (day 45).

To detect and quantify CD8+ OVA specific T cells, a MHC-pentameric assay was used. Whole blood samples were collected into heparinized tubes. Whole blood was diluted in PBS EDTA 2mM and layered on lympholyte M separation cell media (TEBU BIO #CL5031), then centrifuged 1500xg 20 min at room temperature without brake. The enriched cell fraction of splenocytes at interphase was collected and transferred into 96 wells plate to proceed with the surface staining. Cells were washed with wash buffer (0.1% BSA in PBS) by centrifugation 5 min at 400xg. Pellets were resuspended in wash buffer containing 10 µL/test of R-PE Pro MHC Pentamer H-2Kb SIINFEKL (ProImmune #F093-2B-E) and incubated at +4°C for 30 min. Cells were washed with wash buffer (0.1% BSA in PBS) by centrifugation 5 min at 400xg. Pellets were resuspended in wash buffer containing CD8 APC (BD Pharmingen #553035) and CD19 V450 (BD Pharmingen #560376) and incubated for 30 min at +4°C. Cells were washed with wash buffer (0.1% BSA in PBS) by centrifugation 5 min at 400xg. Pellets were resuspended in 100 µL MACSQuant Running Buffer for flow cytometry analysis. The Pentamer-positive cells were viewed by gating first on CD19 negative lymphoid cells and then analyzing on a two-color plot showing CD8 vs Pentamer (**Figure 8**).

At day 14, it is shown that more antigen specific CD8+ T cells are displayed with the PCPTG19874 vector than with the PCPTG19873 vector.

EXAMPLE 9: Comparison of PCPV-polyepitopes vs PCPVpolyepitopes-mIL12 in the E.G7-OVA model

We compared the antitumor efficacy of PCPV encoding tumor epitopes (PCPTG19873) to PCPV encoding tumor epitopes and mIL12 (PCPTG19874) in the murine T lymphoblast cell line E.G7-OVA (ATCC CRL-2113), a cell line known to express the epitopes encoded in PCPTG19873 and

PCPTG19874 vectors. C57BL/6 mice were grafted with $3x10^5$ cells sc. Day 5, 12 and 19, $1x10^7$ pfu of PCPV-ctr, PCPTG19873 or PCPTG19874 were injected into the growing tumors (10 mice per group). Tumor growth and survival proportions were monitored.

Treatment with both PCPV vaccines increased survival frequencies ($p<0.05$). Furthermore, animals treated with the PCPV expressing IL12 (PCPTG19874) had significantly lower tumor sizes than animals treated with the PCPV expressing antigen only ($p<0.05$ at day 26). This observation demonstrates the therapeutic added value of the recombinant IL12 encoded within the PCPV vector (**Figure 9**).

EXAMPLE 10: Detection of murine IL12 in extracellular vesicle (EV) fraction isolated from infected PBMCs.

PBMCs from a healthy donor were cultivated in RPMI, supplemented with 1% glutamine and 1% gentamicin, and 10% fetal bovine serum (FBS). The serum was pretreated by ultracentrifugation o/n at 100 000 g to eliminate serum inherent EVs.

$1x10^8$PBMCs were infected in 35 ml with PCPV-mIL12 (PCPTG19818) at the MOI of 1, or virus was added to cell-free medium. Next day, the cultures were centrifuged at 300 g, then 2000 g. The cleared supernatants were concentrated using a Centricon 70-Plus, membrane Ultracel-PL, 10 kD (Merck Millipore) to a volume of 2 ml. This solution was filtered using Ultrafree-CL Centrifugal Filter 0.1 µm pore size (Merck Millipore). Next, 11 ml of PBS were added, and the solution was ultracentrifuged in 14 mL, Open-Top Thinwall Ultra-Clear Tube, 14 x 95mm - 50Pk (Beckman) o/n at 100000g. The pellet, corresponding to the EV fraction, was dissolved in 100 µl of PBS.

The mIL12 was quantified in various fractions of the EV isolation procedure by Luminex (mIL12 Simplex) (**Figure 10)**. Compared to the cell-free control, mIL12 increased in the various fractions. This observation suggests that virus infection and mIL12 gene expression occurred in infected PBMC cultures.

Unexpectedly, mIL12 was readily detected in the EV fraction. This suggests that mIL12 was associated with EVs and such modified EVs associated with IL-12 can exert activation of target cells such as, but not limited to, pDCs, NK, B cells.

EXAMPLE 11: Immunogenicity of PCPV-OVA vs PCPV-OVA-mIL12

Here we probed the immunogenicity of PCPV-OVA vectors, encoding IL12 (PCPTG19874) or not (PCPTG19873), by intracellular cytokine staining (ICS): $1x10^7$ pfu of a PCPV ctr vector, wherein said polyepitopes encoding vectors PCPTG19873 and PCPTG19874 were injected days 0, 7 and 14 into

the flank (subcutaneously) of naïve C57BL/6 mice. After 45 days, mice were sacrificed, and splenocytes were isolated. Frozen splenocytes were thawed, cultivated, and stimulated for 5 hours in the presence of OVA specific peptide SIINFEKL or appropriate controls. The percentage of live CD8+ T cells secreting IFN-gamma is shown in **Figure 12**.

Splenocytes (1x10E^6 cells), harvested from three different immunized mice were incubated for 4-6 hours with 1µg/ml OVA peptide (ProteoGenix NH2-SIINFEKL-COOH) or irrelevant peptide (ProteoGenix NH2-KNGENAQAI-COOH) in RPMI 1640 media containing 10 % FBS. Golgi Stop (BD #554724) and Golgi Plug (BD #555029) were added to block cytokine release. Then, splenocytes were stained with surface antibodies CD3 PerCP Cy5.5 (BD Pharmingen #551163) and CD8 V500 (BD Horizon #560776) and with Live Dead Violet (Invitrogen #L34958) for viability, in phosphate-buffered saline (PBS) 20 min at +4°C. Splenocytes were then treated with fixation buffer and permeabilization buffer following the manufacturer's protocol (Becton Dickinson Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit # 554714) and intracellularly stained for interferon gamma (IFNγ-FITC (BD Pharmingen #554411) 30 min at +4°C. Cells were washed with PBS and suspended in 100 µL MACSQuant Running Buffer for flow cytometry analysis. To determine the percentage of CD3+CD8+IFNγ+ cells amongst the population, a following gating strategy with Kaluza software 2.1.1 (Beckman Coulter) was used: Cells were identified by side scatter forward scatter (SSC vs FSC). From this gate, a dot plot (Live Dead Violet vs SSC) was created to determine the Live cells. From the Live Dead Violet negative cells, a dot plot CD8 vs CD3 was created to gate on CD3+ CD8+ cells. From this gate, a dot plot CD8 vs IFNγ allowed to target CD3+CD8+IFNg+ cells. As it can be assessed on **Figure 12**, results show that the highest percentage IFN-gamma secreting CD8+ T cells were detected after stimulation with the OVA-peptide SIINFEKL in splenocytes from mice vaccinated with PCPTG19874 (PCPV-OVA-IL12). Compared to PCPTG19873 (PCPV-OVA), PCPTG19874 (PCPV-OVA-IL12) induces an IFN-gamma secreting CD8+ T cells response at least as twice superior.

EXAMPLE 12: Comparison of therapeutic vaccination with PCPV-OVA and PCPV-OVA-IL12 versus MVA-OVA and MVA-OVA-IL12 after repeated subcutaneous injections

PCPV and MVA-based therapeutic vaccines were compared in an OVA-specific tumor model E.G7-OVA. C57BL/6 mice were grafted with 3x10^5 cells sc.

At day 5, 12 and 19, 1x10^7 pfu of PCPV ctr, PCPV-OVA (PCPTG19873), PCPV-OVA-IL12 (PCPTG19874), MVA-ctr, MVA-OVA (MVATG20022) or MVA-OVA-IL12 (MVATG20014) were injected subcutaneously, at 2 cm distance from growing tumor (10 mice per group). Tumor growth and survival proportions were monitored (**Figure 13** and **Figure 14**).

Treatment with all OVA-encoding vectors increased survival and reduced tumor growth. Better survival proportions were obtained with vectors encoding both OVA and IL12, respectively PCPTG19874 for the PCPV based vector and MVATG20014 for the MVA based vector (**Figure 13**). In that sense, PCPV and MVA backbones were comparable. Tumor growth was better controlled after subcutaneous treatment with PCPV-OVA-IL12 compared to PCPV-OVA. Same observation was made for MVA-OVA-IL12 and MVA-OVA.

Surprisingly, tumor control with PCPV-OVA-IL12 was better than with MVA-OVA-IL12 (**Figure 14**). The results validate the use of PCPV for subcutaneous vaccination schemes.

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CLAIMS

1. A recombinant pseudocowpox virus (PCPV) comprising at least one heterologous nucleic acid inserted in its genome;
wherein one of the at least one heterologous nucleic acid inserted in its genome is a nucleic acid encoding for an interleukin 12 (IL-12).

2. The PCPV of claim 1, wherein said PCPV is obtained from the wild-type TJS strain as identified by ATCC reference number ATCC VR-634™ or from a virus strain of the same or similar name or functional fragments and variants thereof.

3. The PCPV of claim 1 or 2, wherein said PCPV is further defective for a viral function encoded by the PCPV genome and preferably is defective for a non-essential viral function and, more preferably for a viral gene function encoded at the insertion site of said heterologous nucleic acid.

4. The PCPV of any one of claims 1 to 3, wherein said heterologous nucleic acid encodes a polypeptide selected from the group consisting of a suicide gene product; an immunomodulatory polypeptide; an antigenic polypeptide; an antibody; a functional derivative of an antibody; a functional fragment of an antibody; and any combination thereof.

5. The PCPV of claim 4, wherein said immunomodulatory polypeptide is selected from the group consisting of cytokines, such as interleukins, chemokines, interferons, tumor necrosis factor, colony-stimulating factors, APC-exposed proteins, polypeptides having an anti-angiogenic effect and agonists or antagonists of immune checkpoints, an any combination thereof.

6. The PCPV of any of claims 4-5, wherein said immunomodulatory polypeptide is an interleukin or a colony-stimulating factor and, in particular GM-CSF or is an agonist OX40-directed antibody.

7. The PCPV of any of claims 4-5, wherein said immunomodulatory polypeptide is a tumor necrosis factor, in particular a CD40L, an IL-4, an IL-21 or a BAFF.

8. The PCPV of claim 4, wherein said antigenic polypeptide is a tumor antigen, selected from the group consisting of tumor-associated antigens (TAA), tumor-specific antigens (TSA) and oncoviral antigens.

9. The PCPV of claim 8, wherein said antigenic polypeptide is a full-length antigen.

10. The PCPV of claim 8 wherein the tumor antigen is a class I or class II epitope derived from a tumor antigen, or a combination of class I epitopes, or a combination of class II epitopes, or a combination of class I and class II epitopes.

11. The PCPV of claim 8, wherein said TAA is selected from the group consisting of WT1, MAGE-A3 MUC1, HER2/Neu and NY-ESO-1; or a tissue specific TAA, preferably a tissue specific TAA selected among, Mesothelin, PSA, and gp100.

12. The PCPV of claim 8, wherein said TSA is selected from the group consisting of viral oncogenes; preferably a viral oncogene inducing virus selected among EBV antigens, HPV antigens; or a mutated self TSA, preferably a mutated self TSA selected among EGFRvIII, KRAS, p53 and BRAF; or neo-epitopes resulting from non-synonymous somatic mutations.

13. The PCPV of claim 8, wherein the oncoviral antigens is selected among viral oncogene inducing virus selected among EBV antigens or HPV antigens.

14. The PCPV of any one of claims 1 to 13, wherein the at least one heterologous nucleic acid is operably linked to suitable regulatory elements for expression in a desired host cell or subject.

15. The PCPV of claim 4, wherein the at least one heterologous nucleic acid is placed under the control of a poxvirus promoter, preferably, a vaccinia virus promoter and more preferably one selected from the group consisting of the p7.5K, pH5R, p11K7.5, pSE, pTK, pB2R, p28, p11, pF17R, pA14L, pSE/L, pA35R, pC1R and pK1L promoter, synthetic promoters and early/late chimeric promoters.

16. The PCPV of any one of claims 1 to 15, wherein the at least one heterologous nucleic acid is inserted in the VEGF locus.

17. The PCPV of any one of claims 1 to 16, wherein the IL-12 is human IL-12.

18. The PCPV of any one of claims 1 to 17, wherein the IL-12 is a fusion protein comprising an IL-12 p40 subunit and an IL-12 p35 subunit.

19. The PCPV of claim 18, wherein the IL-12 p40 subunit is N-terminal to the IL-12 p35 subunit.

20. The PCPV of claim 18 or 19, wherein the IL-12 p40 subunit comprises the amino acid sequence of SEQ ID NO: 1 or an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the amino acid sequence of SEQ ID NO: 1.

21. The PCPV of any one of claims 18-20, wherein the IL-12 p35 subunit comprises the amino acid sequence of SEQ ID NO: 2 or an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the amino acid sequence of SEQ ID NO: 2.

22. The PCPV of any one of claims 18-21, wherein the IL-12 p40 subunit and the IL-12 p35 subunit are fused in a single polypeptide via an amino acid linker.

23. The PCPV of claim 22, wherein said amino acid linker is about 5 to about 10 amino acids in length.

24. The PCPV of claim 22 or 23, wherein said amino acid linker is 7 amino acids in length.

25. The PCPV of any one of claims 22-24, wherein the amino acid linker is a glycine-serine linker.

26. The PCPV of any one of claims 22-25, wherein the amino acid linker comprises the amino acid sequence of SEQ ID NO: 3.

27. The PCPV of any one of claims 1-26, wherein the IL-12 comprises the amino acid sequence of SEQ ID NO: 4 or an amino acid sequence that is at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the amino acid sequence of SEQ ID NO: 4.

28. The PCPV of any one of claims 18-21, wherein the IL-12 p40 subunit and the IL-12 p35 subunit are directly fused in a single polypeptide.

29. The PCPV of any one of claims 1-26, wherein the nucleic acid sequence encoding the IL-12 comprises a nucleotide sequence at least 75% identical, at least 80% identical, at least 85% identical, at least 90% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, or at least 99% identical to the nucleotide sequence of SEQ ID NO: 5.

30. The PCPV of claim 29, wherein the nucleic acid sequence encoding the IL-12 comprises the nucleotide sequence of SEQ ID NO: 5.

31. The PCPV of any one of claims 1-30, wherein the nucleic acid sequence encoding the IL-12 is inserted within the VEGF locus.

32. The PCPV of any one of claims 1-31, wherein the nucleic acid sequence encoding the IL-12 is inserted within the VEGF locus; and said PCPV further comprises a heterologous nucleic acid encoding for an antigenic polypeptide which is also inserted within the VEGF locus.

33. The PCPV of claim 32, wherein the expression cassette comprising the nucleic acid sequence encoding the IL-12 and the expression cassette comprising the nucleic acid encoding the antigenic polypeptide are in a back-to-back orientation in relation to each other within the VEGF locus.

34. The PCPV of any one of claims 32-33, wherein the nucleic acid sequence encoding the IL-12 is placed under control of a pSE/L promoter, and the nucleic acid encoding the antigenic polypeptide is placed under control of a p7.5K promoter.

35. A method for generating the PCPV of anyone of claims 1 to 34, by homologous recombination between a transfer plasmid comprising the heterologous nucleic acid flanked in 5' and 3' with PCPV sequences respectively present upstream and downstream the insertion site and a PCPV genome, wherein said method comprises a step of generating said transfer plasmid and a step of introducing said transfer plasmid into a suitable host cell, notably together with a PCPV virus comprising the flanking sequence present in the transfer plasmid.

36. The method according to claim 35, wherein the site of insertion of the at least one heterologous nucleic acid in the PCPV genome is in a viral gene, with a preference for a non-essential viral gene, in an intergenic region, in a portion of the PCPV genome which does not encode gene products or in a duplicated locus.

37. The method according to claim 36, wherein upon insertion of the heterologous nucleic acid in the PCPV genome the viral locus at the insertion site is deleted at least partially, resulting in a defective PCPV virus for said virus function.

38. The method according to claim 36 or 37, wherein said at least one heterologous nucleic acid is inserted in the VEGF locus.

39. The method according to anyone of claims 35 to 38, wherein the transfer plasmid further comprises one or more selection and/or detectable gene to facilitate identification of the recombinant PCPV.

40. The method according to claim 39, wherein the transfer plasmid is introduced into the host cell in the presence of an endonuclease capable of providing a double-stranded break in said selection or detectable gene.

41. The method according to claim 39 or 40, wherein the selection gene is the GPT gene encoding a guanine phosphoribosyl transferase permitting growth in a selective medium and/or said detectable gene encodes GFP, e-GFP or mCherry.

42. The method according to anyone of claims 35 to 41, wherein said suitable host cell is selected from Bovine Turbinate (BT) cell or HeLa cell.

43. A method for amplifying the PCPV according to anyone of claims 1 to 34 or generated by the method according to anyone of claims 35 to 42, comprising the steps of a) preparing a producer cell line, b) transfecting or infecting the prepared producer cell line, c) culturing the transfected or infected producer cell line under suitable conditions so as to allow the production of the virus, d) recovering the produced virus from the culture of said producer cell line and optionally e) purifying said recovered virus.

44. The method of claim 43, wherein said producer cell is Bovine Turbinate (BT) or HeLa.

45. A composition comprising a therapeutically effective amount of the PCPV according to anyone of claims 1 to 34 or amplified by the method according to anyone of claims 43 to 44 and a pharmaceutically acceptable vehicle.

46. The composition of claim 45, wherein said composition is formulated in individual doses comprising from approximately 10^3 to approximately 10^{12} pfu, advantageously from approximately 10^4 pfu to approximately 10^{11} pfu, preferably from approximately 10^5 pfu to approximately 10^{10} pfu; and more preferably from approximately 10^6 pfu to approximately 10^9 pfu of PCPV.

47. The composition of claim 45 or 46, wherein the composition is formulated for intravenous, intramuscular, intradermal, intranasal, subcutaneous or intratumoral administration.

48. The composition of anyone of claims 45 to 47, for use for treating or preventing diseases or pathological condition caused by a pathogenic organism or an unwanted cell division, or for inhibiting tumor cell growth.

49. A method of treatment comprising administering the composition of anyone of claims 45 to 48 to a subject in need thereof in an amount sufficient to treat or prevent a disease or a pathological condition caused by a pathogenic organism or an unwanted cell division.

50. A method for inhibiting tumor cell growth comprising administering the composition of anyone of claims 45 to 48 to a subject in need thereof.

51. The composition for use or the method according to any one of claims 48 to 50, comprising 2 to 6 weekly administrations possibly followed by 2 to 15 administrations at 3 weeks interval of the PCPV composition comprising 10^6 to 10^9 pfu.

52. The composition for use or the method according to any one of claims 48 to 51, wherein said method or use is for treating a cancer selected from the group consisting of renal cancer, prostate cancer, breast cancer, bladder cancer, colorectal cancer, lung cancer, liver cancer, gastric cancer, bile duct carcinoma, endometrial cancer, pancreatic cancer, ovarian cancer, head and neck cancer, melanoma, glioblastoma, multiple myeloma, or malignant glioma cells.

53. The composition for use or the method according to anyone of claims 48 to 52, which is used in conjunction with one or more other therapeutic agents selected from the group consisting of surgery, radiotherapy, chemotherapy, cryotherapy, hormonal therapy, toxin therapy, immunotherapy, cytokine therapy, targeted cancer therapy, gene therapy, photodynamic therapy and transplantation.

54. The composition for use or the method according to claim 53, which is carried out according to a prime boost approach which comprises sequential administrations of a priming composition(s) and a boosting composition(s).

55. The composition for use or the method according to claim 54, wherein the priming composition is a PCPV composition, and the boosting composition is a MVA composition or a PCPV composition.

56. The composition for use or the method according to claim 55, whereas the priming composition is a PCPV composition administered by intratumoral route and the boosting composition is a MVA composition administered by intravenous route.

57. A method for eliciting or stimulating and/or re-orienting an immune response comprising administering the composition according to anyone of claims 45 to 48 to a subject in need thereof, in an amount sufficient to activate the subject's immunity.

58. A method according to claim 57, resulting in at least one the following properties:

- The secretion of high levels of IFN-alpha from PBMC;
- The activation of monocyte-derived dendritic cells;
- The induction of T cell activation or proliferation;
- A better cytokine/chemokine profile in MDSC;
- Activation of APC;
- A M2 to M1 conversion of human macrophages; and/or
- Induction of immunity through a TLR9-mediated pathway or others innate immunity-stimulating pathways.

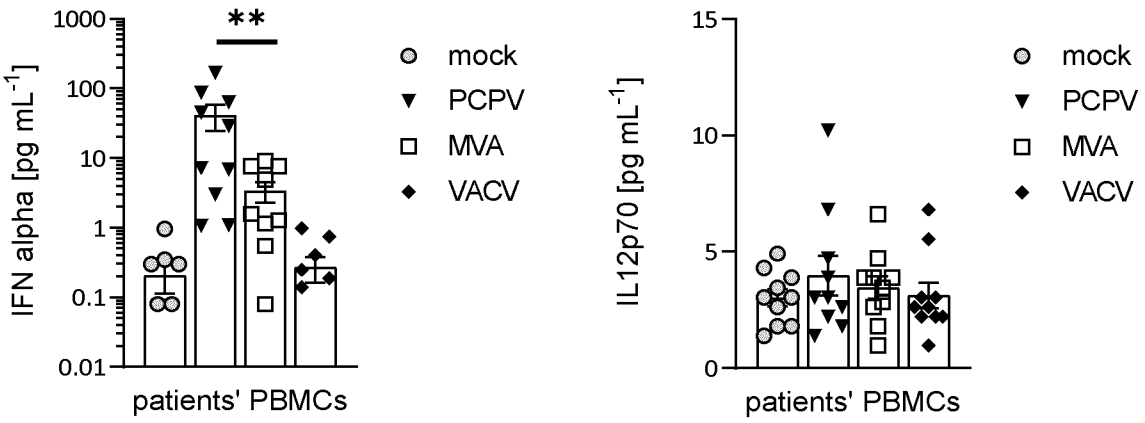

**
1000
100
10
1
0.1
0.01
IFN alpha [pg mL-1]
patients' PBMCs
mock
PCPV
MVA
VACV
15
10
5
0
IL12p70 [pg mL-1]
patients' PBMCs
mock
PCPV
MVA
VACV

Figure 1

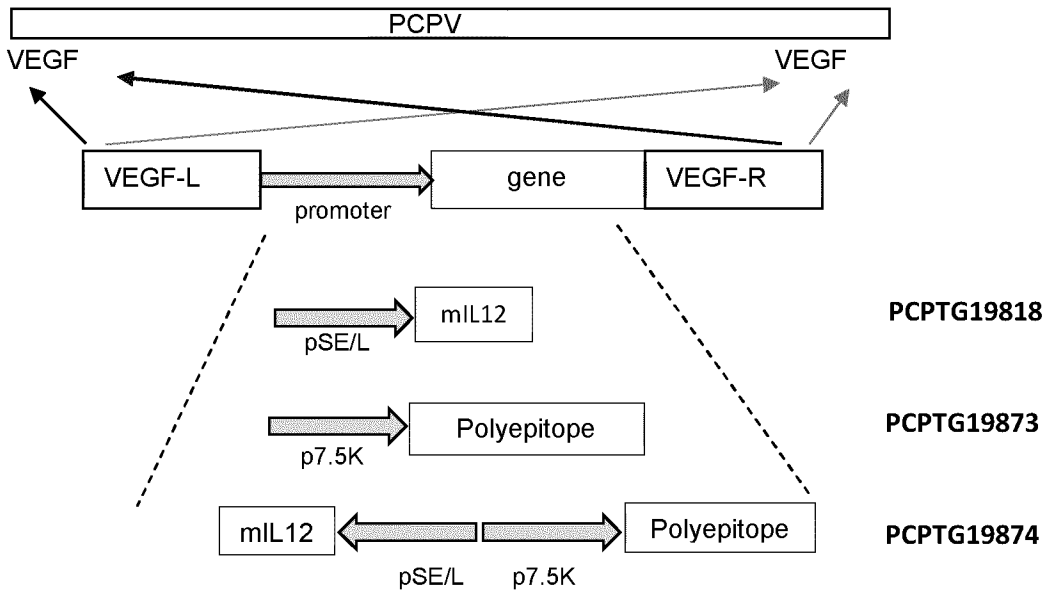

PCPV
VEGF
VEGF
VEGF-L
promoter
gene
VEGF-R
mIL12
pSE/L
PCPTG19818
Polyepitope
p7.5K
PCPTG19873
mIL12
Polyepitope
pSE/L
p7.5K
PCPTG19874

Figure 2

Fig. 3A

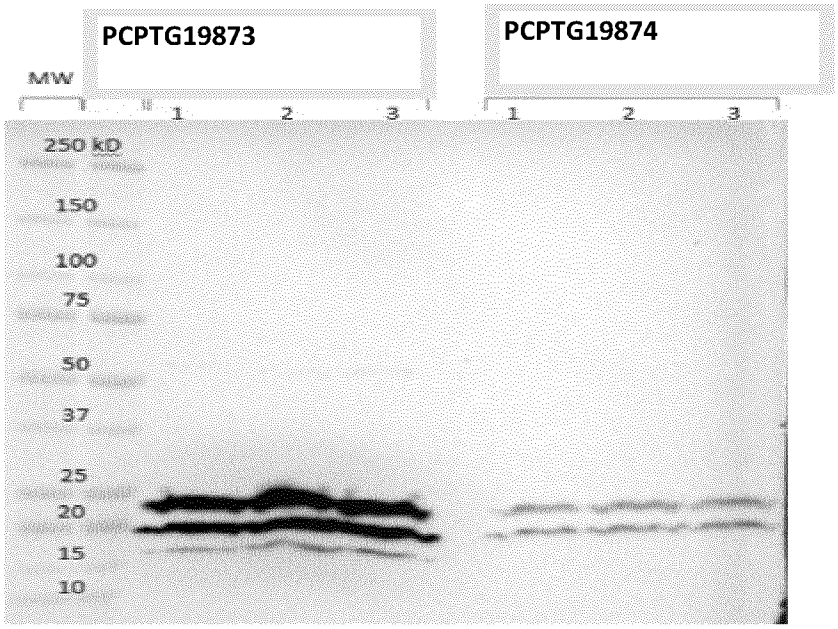


Fig. 3B

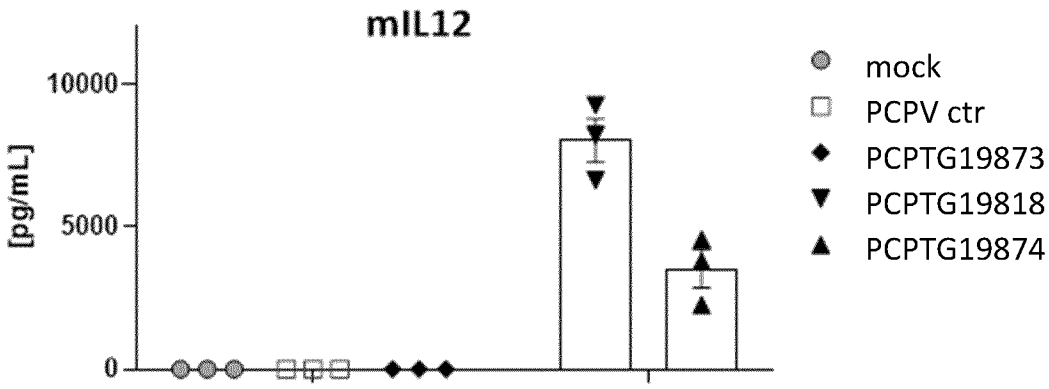


Figure 3

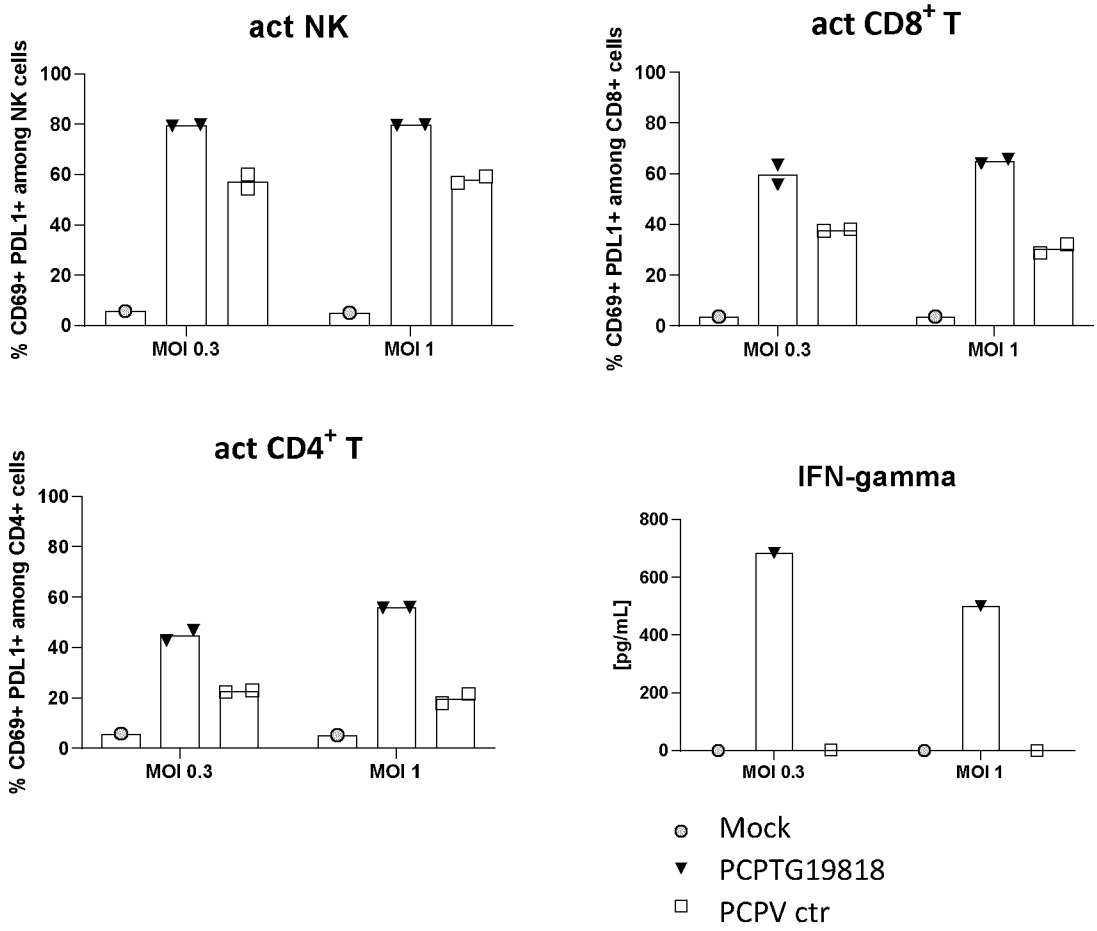

act NK
% CD69+ PDL1+ among NK cells
100
80
60
40
20
0
MOI 0.3
MOI 1
act CD8+ T
% CD69+ PDL1+ among CD8+ cells
act CD4+ T
% CD69+ PDL1+ among CD4+ cells
IFN-gamma
[pg/mL]
800
600
400
200
Mock
PCPTG19818
PCPV ctr

Figure 4

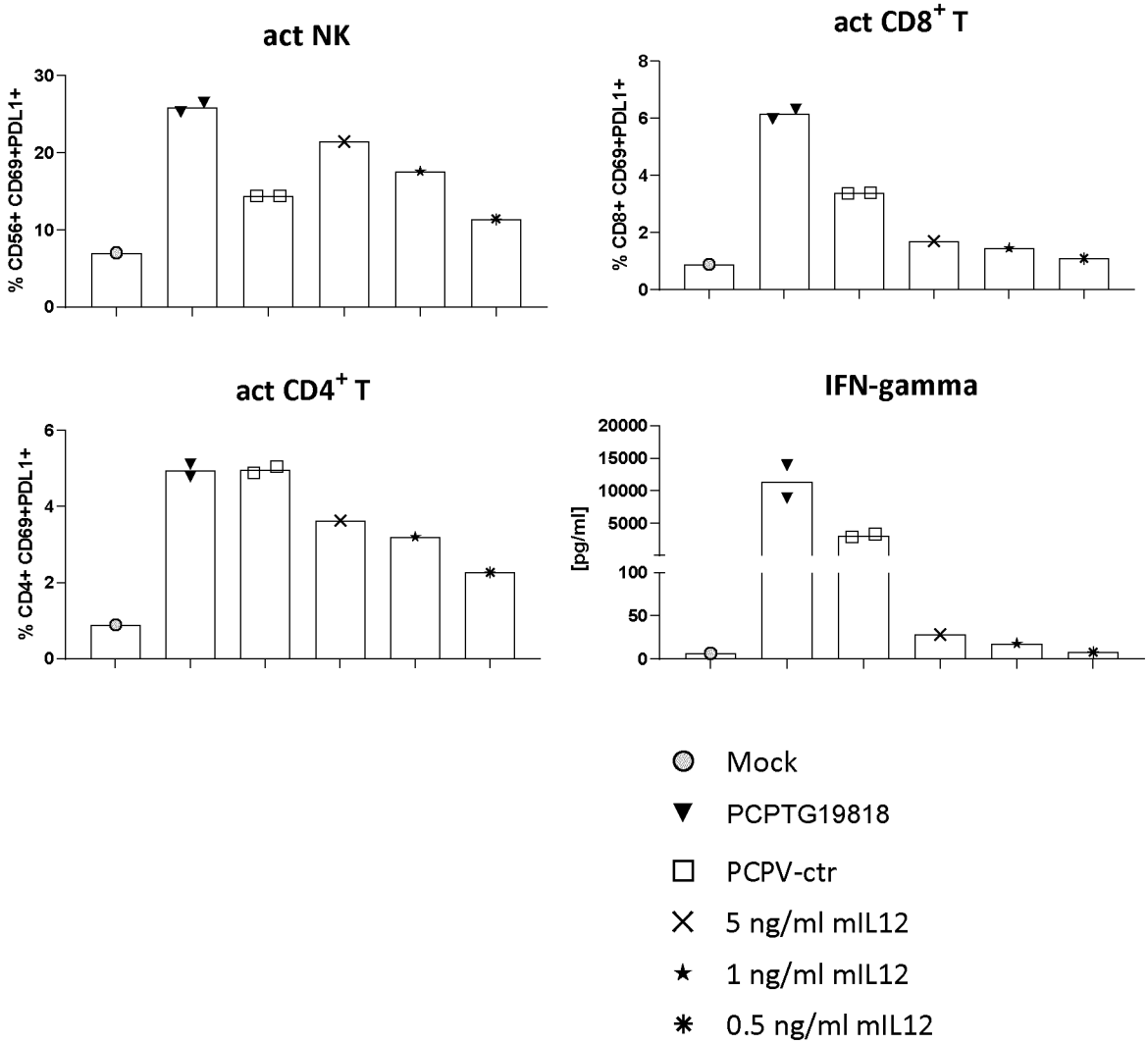

act NK
% CD56+ CD69+PDL1+
30
20
10
0
act CD8+ T
% CD8+ CD69+PDL1+
8
6
4
2
0
act CD4+ T
% CD4+ CD69+PDL1+
6
4
2
0
IFN-gamma
[pg/ml]
20000
15000
10000
5000
100
50
0
Mock
PCPTG19818
PCPV-ctr
5 ng/ml mIL12
1 ng/ml mIL12
0.5 ng/ml mIL12

Figure 5

Fig. 6A

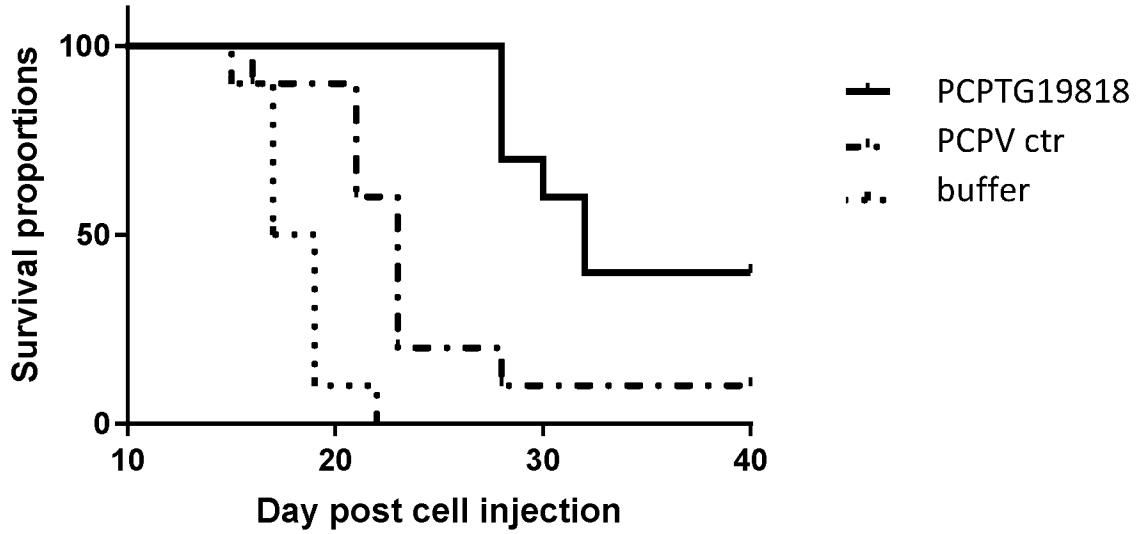


Fig. 6B

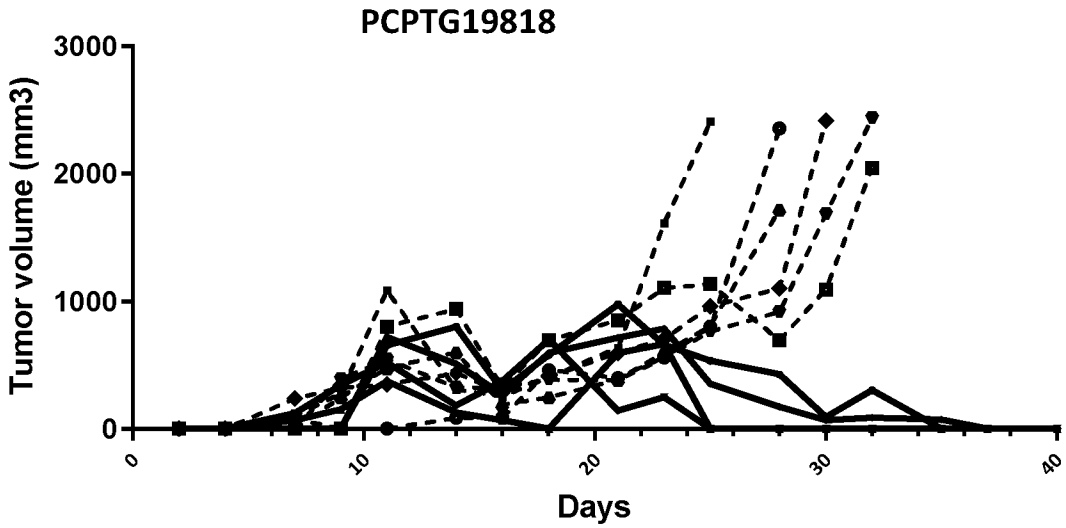


Figure 6

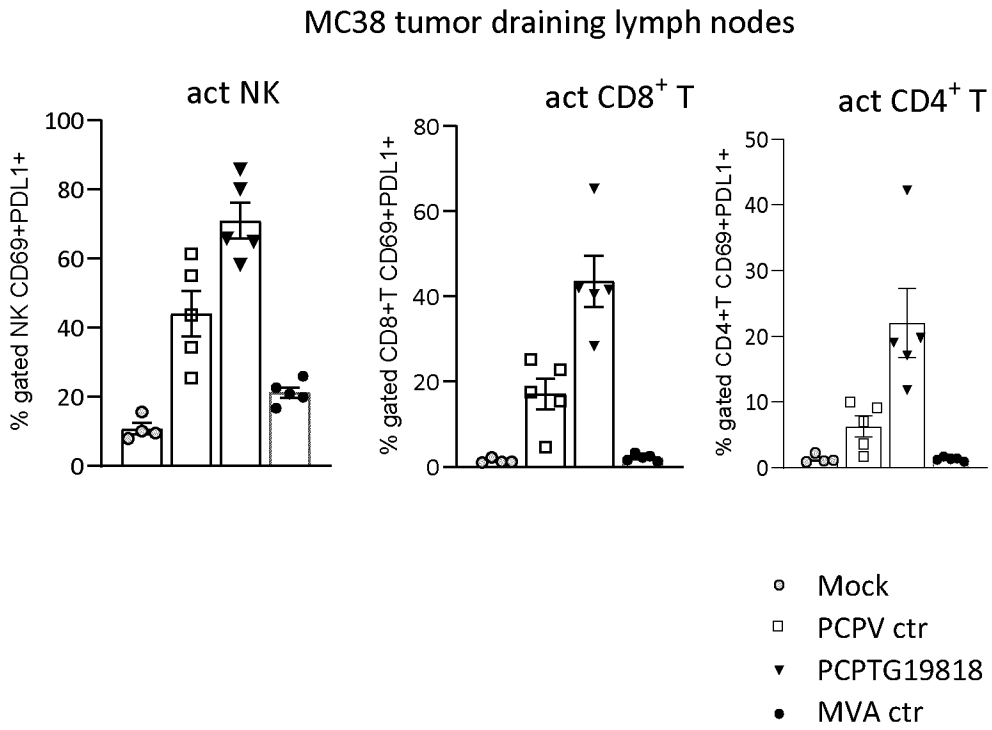


Figure 7

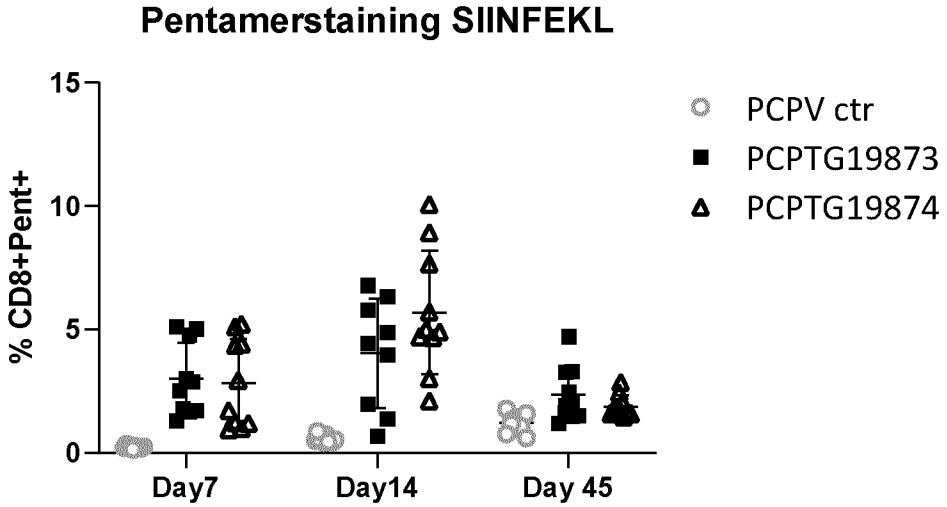
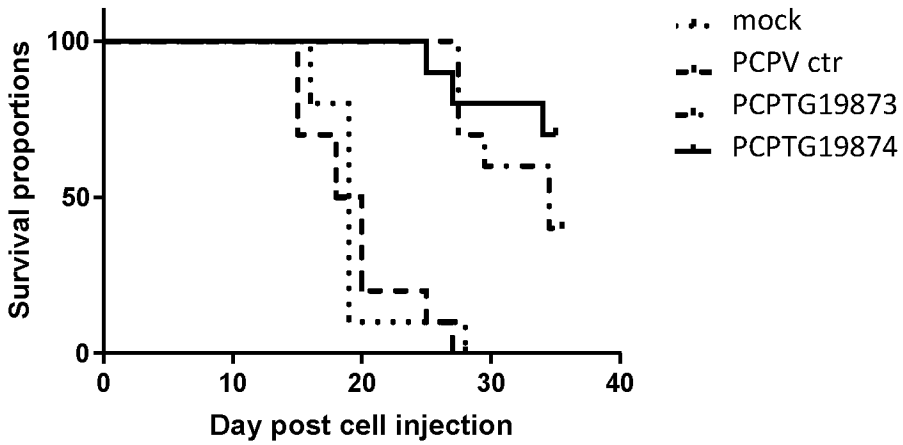


Figure 8


Survival proportions
100
50
0
0
10
20
30
40
Day post cell injection
mock
PCPV ctr
PCPTG19873
PCPTG19874

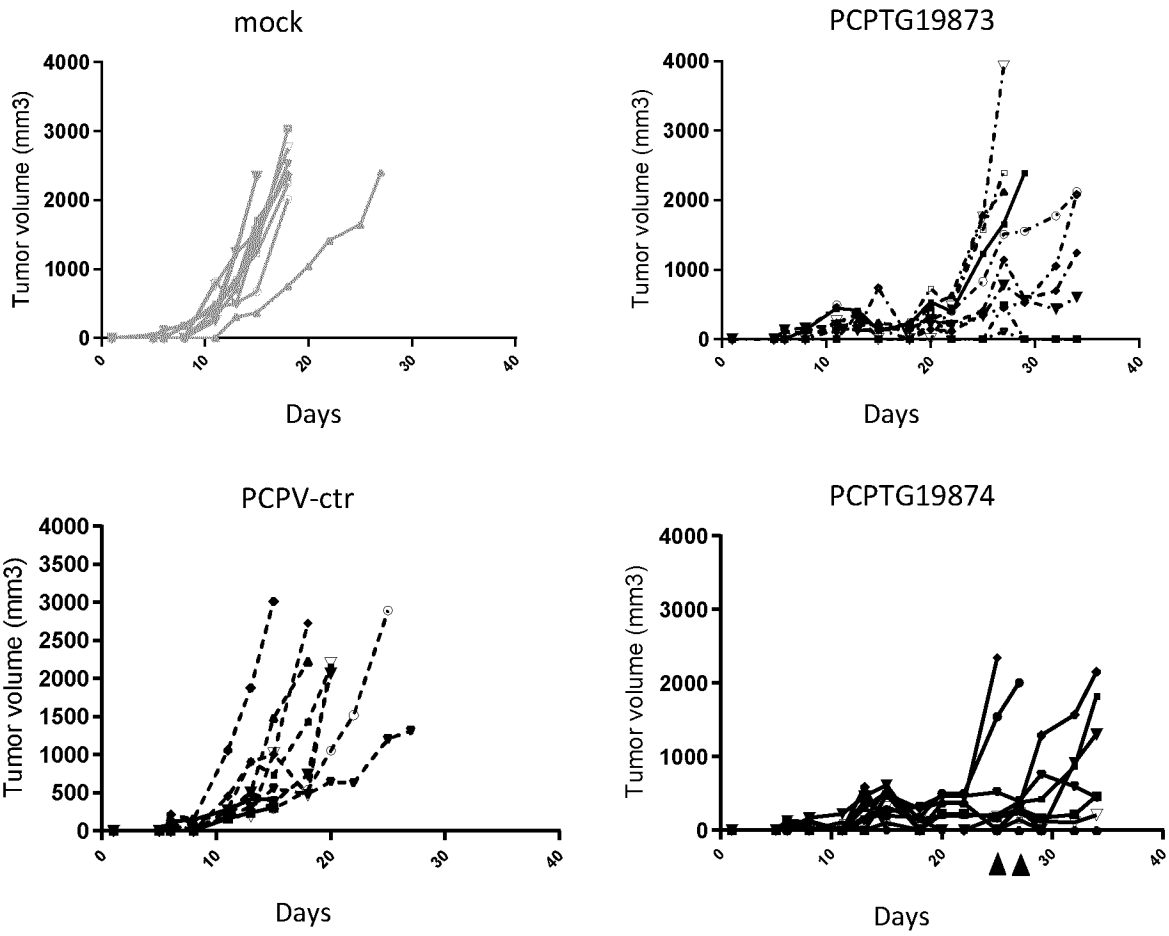

mock
PCPTG19873
PCPV-ctr
PCPTG19874
Tumor volume (mm3)
Days
4000
3000
2000
1000
0
3500
2500
1500
500
10
20
30
40

Figure 9

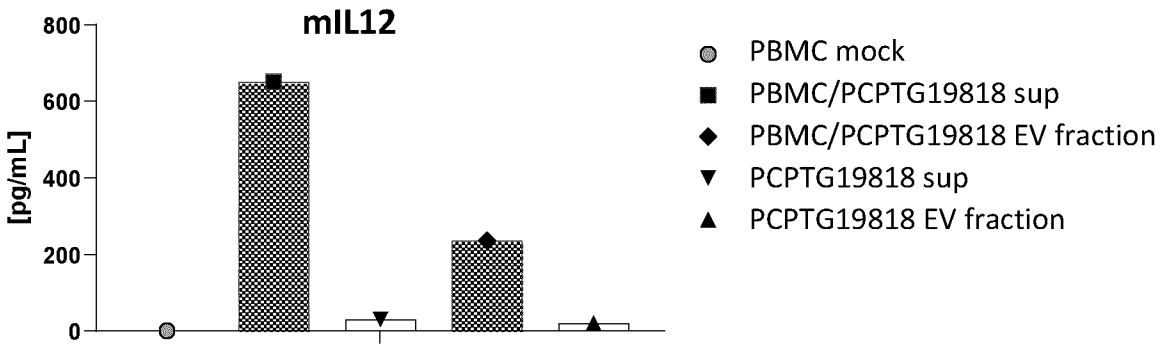


Figure 10

Figure 11

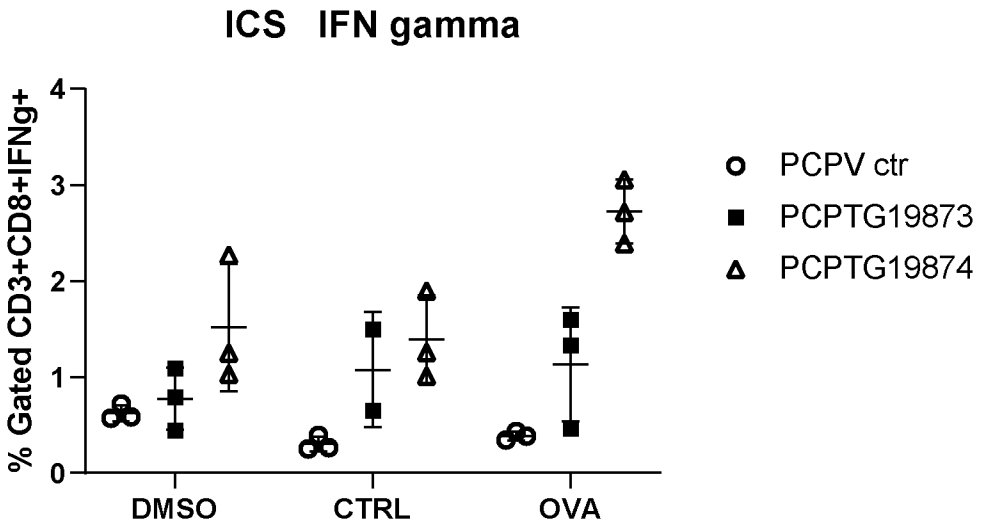
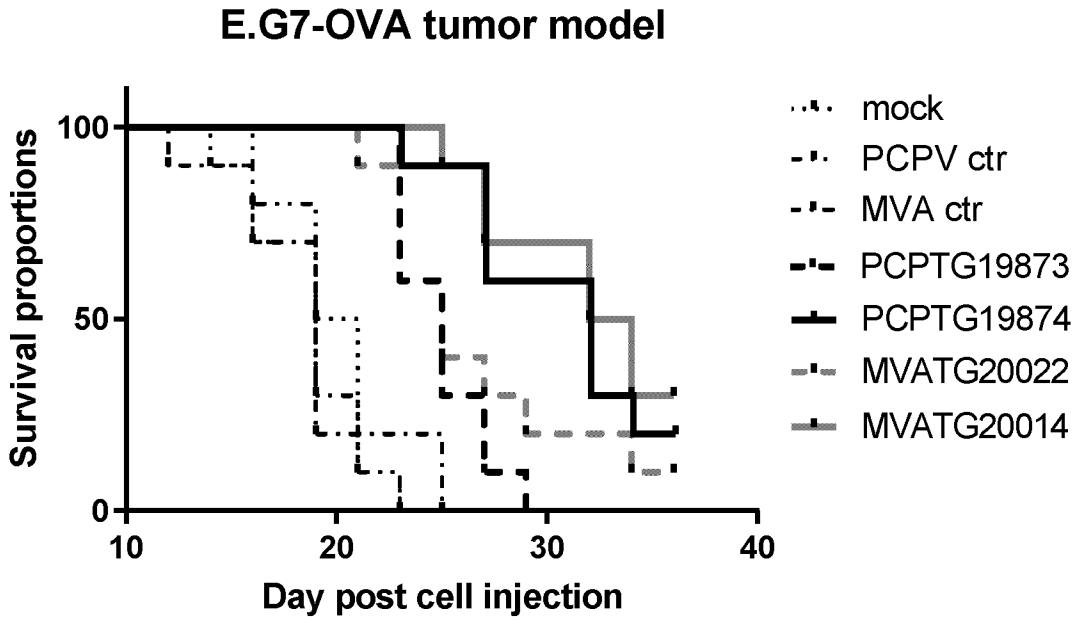


Figure 12



Treatment: Day 5, 12 and 19: $1x10^7$ pfu **sc**, same flank as tumor.

Figure 13

Fig. 14A

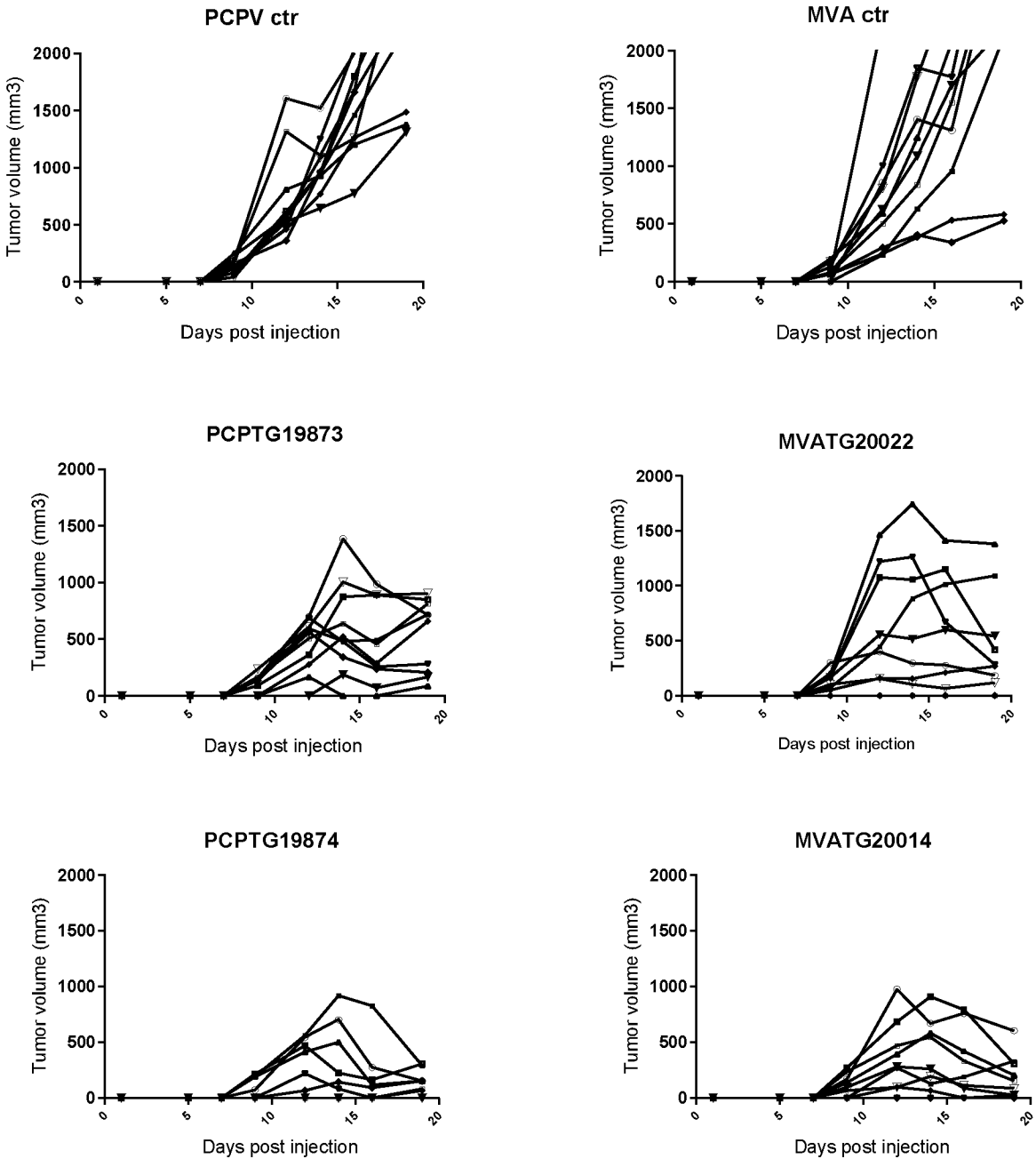


Figure 14

Fig. 14B

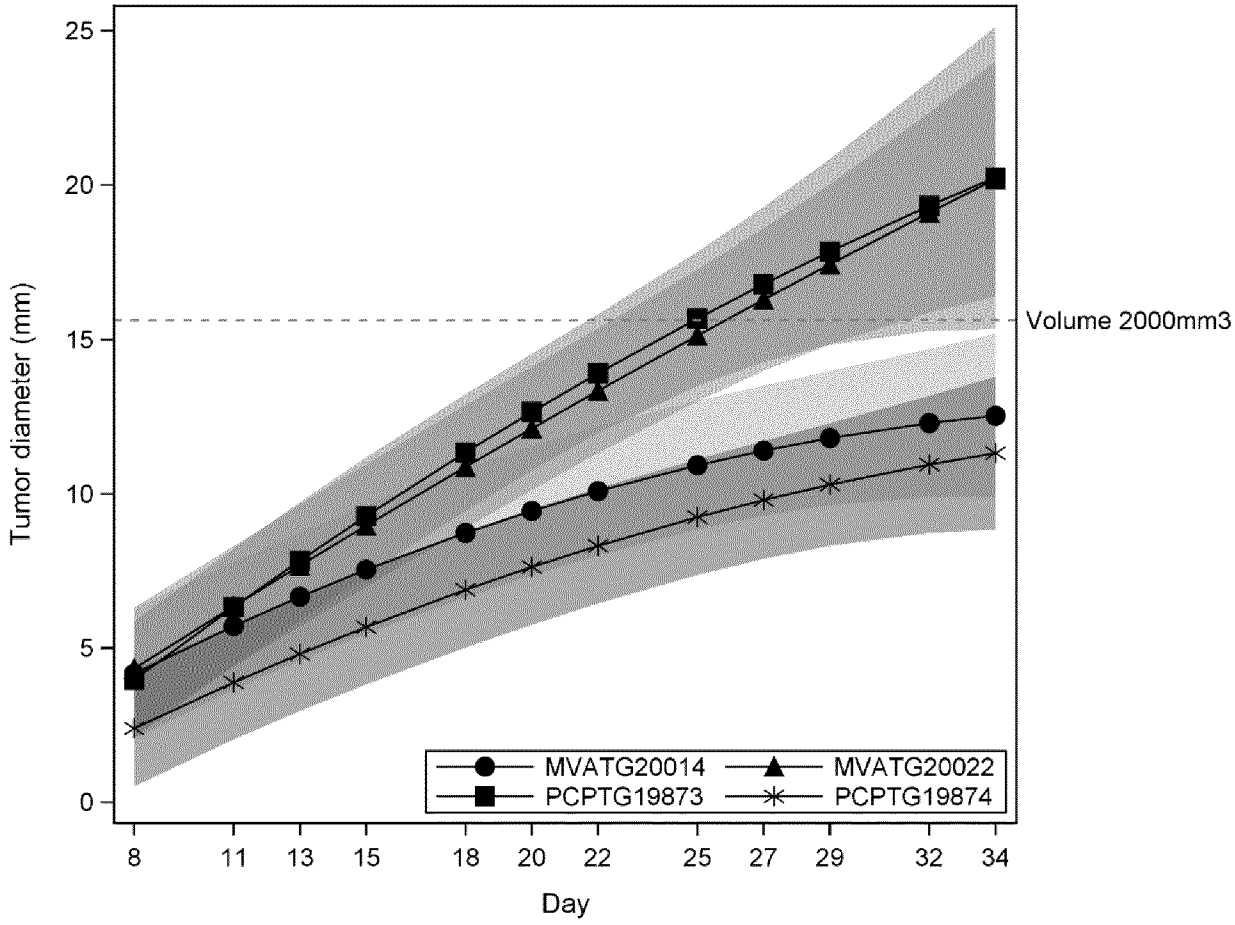


Figure 14 (Continued)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/076224

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/54 A61K35/00 A61K39/00 C12N15/86
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2019/170820 A1 (TRANSGENE [FR]) 12 September 2019 (2019-09-12) cited in the application page 14, line 18 - line 23; claims; examples -----	1-6, 14-16, 31,35-58 1-58
Y	RAMOS R N ET AL: "Pseudocowpox virus, a novel vector to enhance the therapeutic efficacy of antitumor vaccination", CLINICAL & TRANSLATIONAL IMMUNOLOGY, vol. 11, no. 5, 1 January 2022 (2022-01-01), XP093107453, GB ISSN: 2050-0068, DOI: 10.1002/cti2.1392 Retrieved from the Internet: URL:https://onlinelibrary.wiley.com/doi/full-xml/10.1002/cti2.1392> abstract, results, discussion, figures; -----	1-58

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 2023	21/12/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Sommer, Birgit

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2018/209194 A2 (ICAHN SCHOOL MED MOUNT SINAI [US] ET AL.) 15 November 2018 (2018-11-15) claims; sequence 43	1-58
Y	WO 2018/234506 A2 (TRANSGENE SA [FR]) 27 December 2018 (2018-12-27) cited in the application page 10, line 21 - line 32; claims; examples	1-58
Y	WO 2017/060360 A1 (INVECTYS [FR]) 13 April 2017 (2017-04-13) claims; examples	1-58

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/076224

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed.

   b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      ☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/076224

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2019170820 A1	12-09-2019	AU 2019229653 A1	24-09-2020
		BR 112020018117 A2	22-12-2020
		CA 3093093 A1	12-09-2019
		CN 112512560 A	16-03-2021
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		US 2021000937 A1	07-01-2021
		WO 2019170820 A1	12-09-2019
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		BR 112019023758 A2	09-06-2020
		CA 3061770 A1	15-11-2018
		CL 2019003233 A1	17-07-2020
		CN 111246877 A	05-06-2020
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		EC SP19080602 A	30-11-2019
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		MA 49298 A	11-05-2018
		PE 20200496 A1	04-03-2020
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		CA 3067405 A1	27-12-2018
		CN 111065406 A	24-04-2020
		EP 3641803 A2	29-04-2020
		IL 271558 A	27-02-2020
		JP 7334124 B2	28-08-2023
		JP 2020530437 A	22-10-2020
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		KR 20200026894 A	11-03-2020
		RU 2020101489 A	21-07-2021
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