

COMPOSITIONS AND METHODS FOR ENHANCING THE STABILITY OF TRANSGENES IN POXVIRUSES

FIELD OF THE INVENTION

[001] The present invention relates to recombinant poxviruses and compositions thereof that comprise a modified Mucin 1, cell surface associated (MUC1) transgene, a human carcinoembryonic antigen (CEA) transgene, and/or one or more costimulatory molecules. In at least one aspect, the modified MUC1, CEA, and/or costimulatory molecule transgenes improve the stability to the poxvirus through successive passaging of the recombinant poxvirus. In additional aspects, the present invention relates to recombinant poxviruses and compositions thereof for use as vaccines and medicinal compositions.

BACKGROUND OF THE INVENTION

[002] Recombinant poxviruses have been used as immunotherapy vaccines against infectious organisms and, more recently, against tumors. Mastrangelo et al. *J Clin Invest.* 2000;105(8):1031–1034. Two of these poxvirus groups, avipoxvirus and orthopoxvirus, have been shown to be effective at battling tumors and have been involved with potential cancer treatments. *Id.*

[003] One exemplary avipoxvirus species, fowlpox, has been shown to be a safe vehicle for human administrations as fowlpox virus enters mammalian cells and expresses proteins, but replicates abortively. Skinner et al. *Expert Rev Vaccines.* 2005 Feb;4(1):63-76. Additionally, the use of fowlpox virus as a vehicle for expression is being evaluated in numerous clinical trials of vaccines against cancer, malaria, tuberculosis, and AIDS. *Id.*

[004] Vaccinia, the most well-known of the orthopoxviruses, was used in the world-wide eradication of smallpox and has shown usefulness as a vector and/or vaccine. Recombinant Vaccinia Vector has been engineered to express a wide range of inserted genes, including several tumor associated genes such as p97, HER-2/neu, p53 and ETA (Paoletti, et al., 1993).

[005] One poxviral strain that has proven useful as an immunotherapy vaccine against infectious disease and cancer is the Modified Vaccinia Ankara (MVA) virus. MVA was generated by 516 serial passages on chicken embryo fibroblasts of the Ankara strain of vaccinia virus (CVA) (for review see Mayr, A., et al. *Infection* 3, 6-14 (1975)). As a consequence of these long-term passages, the genome of the resulting MVA virus had about 31 kilobases of its genomic sequence deleted and, therefore, was described as highly host cell restricted for replication to avian cells (Meyer, H. et al., *J. Gen. Virol.* 72, 1031-1038 (1991)).

It was shown in a variety of animal models that the resulting MVA was significantly avirulent (Mayr, A. & Danner, K., *Dev. Biol. Stand.* 41: 225-34 (1978)).

[006] Strains of MVA having enhanced safety profiles for the development of safer products, such as vaccines or pharmaceuticals, have been described. See International PCT publication WO2002042480 (see also e.g. U.S. Pat. Nos. 6,761,893 and 6,913,752) all of which are incorporated by reference herein. Such variants are capable of reproductive replication in non-human cells and cell lines, especially in chicken embryo fibroblasts (CEF), but are replication incompetent in human cell lines, in particular including HeLa, HaCat and 143B cell lines. Such strains are also not capable of reproductive replication in vivo, for example, in certain mouse strains, such as the transgenic mouse model AGR 129, which is severely immune-compromised and highly susceptible to a replicating virus. See U.S. Pat. Nos. 6,761,893. Such MVA variants and its derivatives, including recombinants, referred to as "MVA-BN," have been described. See International PCT publication WO2002042480 (see also e.g. U.S. Pat. Nos. 6,761,893 and 6,913,752). In the development of cancer immunotherapy vaccines, the tumor antigen MUC1 has been shown to induce and boost a patient's immune response against a variety of cancers when expressed by recombinant poxviruses. See, e.g., Mehebtash et al. *Clin Cancer Res.* 2011 Nov 15;17(22):7164-73.

[007] MUC1 (MUC-1, Mucin 1, cell surface associated) (also known as CD227) is glycoprotein that lines the apical surface of the epithelial cells in the lungs, stomach, intestines, eyes and several other organs and in a small subset of non-epithelial cells such as hematopoietic cells and activated T cells. Its major function in healthy epithelia is to provide lubrication and a physical barrier against chemical and microbial agents. Hollingsworth MA, Swanson BJ (January 2004). "Mucins in cancer: protection and control of the cell surface". *Nature Reviews Cancer* 4 (1): 45–60.

[008] MUC1 is anchored to the apical surface by a transmembrane domain. Hattstrup CL, Gendler, SJ (2008). "Structure and Function of the Cell Surface (Tethered) Mucins". *Annu. Rev. Physiol.* 70: 431–457. The extracellular domain of MUC1 includes a 20 amino acid variable number tandem repeat (VNTR) domain which is usually heavily glycosylated, with the number of repeats varying from 20 to 120 in different individuals. Brayman M, Thathiah A, Carson DD (January 2004). "MUC1: a multifunctional cell surface component of reproductive tissue epithelia". *Reprod Biol Endocrinol* 2: 4.

[009] It has been demonstrated that many human carcinomas (such as ovarian, breast, pancreatic, colorectal, and prostate) and hematologic malignancies (multiple myeloma and some B-cell non-Hodgkin's lymphomas) aberrantly overexpress MUC1. Pecher et al.

Anticancer Res. 2001 Jul-Aug. 21:2591-2596. In contrast to its clustered expression in normal tissues, MUC1 is uniformly distributed over the entire surface of tumor cells. Correa et al. Immunology. Jan 2003; 108(1): 32-41. Moreover, MUC1 is generally underglycosylated in tumors, exposing novel and potentially antigenic epitopes of the protein core to the immune system. Reis et al. Int J Cancer. 1998 Aug 21;79(4):402-10.

[010] In view of MUC1 association with human carcinomas, the prior art has attempted to modify MUC1 in order to enhance immunogenicity of the protein. For example, US2006/0147458 by Hamblin et al., utilized a “codon usage coefficient” in order to design a MUC1 sequence having a reduced homology to native MUC1 as well as having a 7XVNTR segment. US2006/0147458 by Hamblin et al. created a HSP-70-MUC1 fusion protein in an attempt to enhance immunogenicity. US Patent No. 5,744,144, issued to Finn et al., modified a MUC1 protein by adding two 20 amino acid tandem repeats.

[011] Human carcinoembryonic antigen (CEA) is a 180 kD glycoprotein expressed on the majority of colon, rectal, stomach and pancreatic tumors (1), some 50% of breast carcinomas (2) and 70% of lung carcinomas (3). CEA is also expressed in fetal gut tissue, and to a lesser extent on normal colon epithelium. The immunogenicity of CEA has been ambiguous, with several studies-reporting the presence of anti-CEA antibodies in patients (4-7) while other studies have not (8-10). CEA was first described as a cancer specific fetal antigen in adenocarcinoma of the human digestive tract in 1965 (Gold, P. and Freeman, S. O. (1965) Exp. Med. 121:439-462). Since that time, CEA has been characterized as a cell surface antigen produced in excess in nearly all solid tumors of the human gastrointestinal tract. The gene for the human CEA protein has been cloned. (Oikawa et al (1987) Biochim. Biophys. Res. 142:511-518; European Application No. EP 0346710).

[012] There is a substantial, unmet medical need for improving cancer treatments. In view of the effectiveness of the MUC1 and CEA antigens in inducing an immune response against cancers, there is a need for improved vaccines able to effectively introduce the antigens to cancer patients.

[013] In addition, there is an increasing need to provide cancer treatments that are able to successfully overcome the hurdles of seeking regulatory approval. In particular, difficulties with large scale production, impurities, and the like, can be a significant hurdle in obtaining regulatory approval for treatments and translating those treatments to benefiting patients. At least in one aspect, with the development of the various embodiments of the present invention, difficulties involving large scale production, impurities, and other issues have been successfully overcome.

BRIEF SUMMARY OF THE INVENTION

[014] It was determined in the present invention that various substitutions to MUC1, CEA, and/or TRICOM encoding nucleic acids in one or more repetitive nucleotide regions enhance the stability of the MUC1, CEA, and/or TRICOM transgenes in recombinant poxviruses.

[015] Accordingly, in one embodiment, the present invention relates to a recombinant poxvirus which is stable through successive passaging of the recombinant poxvirus. The recombinant poxvirus comprises a first nucleic acid encoding a MUC1 peptide having at least two Variable N-Terminal Repeat (VNTR) domains, wherein a) the arrangement of the at least two VNTR domains are shuffled, and b) the at least two VNTR domains are codon optimized, wherein the recombinant poxvirus is stable through successive passaging.

[016] In one or more preferred embodiments, the recombinant poxvirus comprises a first nucleic acid at least 95% homologous to SEQ ID NO:2 (336 MUC), at least 95% homologous to SEQ ID NO:3 (373 MUC), at least 95% homologous to SEQ ID NO: 4 (399/400 MUC1), or at least 95% homologous to SEQ ID NO: 5 (420 MUC1). In a more preferred embodiment, the recombinant poxvirus comprises a nucleic acid at least 95% homologous to SEQ ID NO: 2 (336 MUC1). In another more preferred embodiment, the recombinant poxvirus comprises a nucleic acid at least 95% homologous to SEQ ID NO:3 (373 MUC).

[017] In yet another preferred embodiment, the recombinant poxviruses further comprises a nucleic acid at least 99% homologous to SEQ ID NOs: 13 or 14 (CEA). In a preferred embodiment, the recombinant poxviruses comprise SEQ ID NOs: 13 or 14.

[018] It is contemplated that the recombinant poxvirus can be any type of poxvirus. In certain embodiments, the poxvirus is an orthopoxvirus or an avipoxvirus. In preferred embodiments, the orthopoxvirus is selected from a vaccinia virus, MVA virus, MVA-BN, and derivatives of MVA-BN. In other more preferred embodiments, the orthopoxvirus is MVA, MVA-BN, or derivatives of MVA-BN. In other preferred embodiments, the avipoxvirus is a fowlpox virus.

[019] In other embodiments, in addition to the MUC1 and/or CEA nucleic acids described herein, the recombinant poxviruses of the present invention include one or more nucleic acids encoding for TRICOM (TRIad of COstimulatory Molecules).

[020] In certain embodiments, the recombinant poxviruses and/or the nucleic acids of the present invention can be used in a heterologous prime-boost dosing regimen. In

preferred embodiments, the regimen comprises: a) one or more priming doses of an MVA virus, the MVA virus including one or more of the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure; and b) one or more boosting doses of a fowlpox virus including one or more of the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure.

[021] It is contemplated that the recombinant poxviruses, nucleic acids, methods, vaccines, and compositions described herein can be embodied in a kit. Accordingly, in a preferred embodiment, the present invention relates to a composition, vaccine, kit, or a use thereof, comprising: a recombinant orthopoxvirus, such as, but not limited to MVA, the recombinant orthopoxvirus including one or more of the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure; and a recombinant avipoxvirus, such as but not limited to fowlpox, including one or more of the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure.

[022] In other embodiments, the present invention relates to one or more methods for generating a recombinant poxvirus encoding for one or more transgenes of the present disclosure that is stable through successive passaging of the recombinant poxvirus.

[023] In one embodiment, there is a method for generating a recombinant poxvirus having a MUC1 transgene that is stable through successive passaging of the recombinant poxvirus, the method comprising: a) providing any one of the nucleic acids or expression cassettes of the present disclosure; and b) inserting the nucleic acid or the expression cassette into a recombinant poxvirus.

[024] In another embodiment, there is a method for generating a recombinant poxvirus that is stable through successive passaging comprising: a) providing a first nucleic acid sequence encoding a MUC1 peptide having at least two Variable N-Terminal Repeat (VNTR) domains, wherein the arrangement of the at least two VNTR domains are shuffled, and the at least two VNTR domains are codon optimized; and b) providing a second nucleic acid encoding a CEA peptide, wherein the second nucleic acid comprises at least one nucleotide substitution in at least one repetitive nucleotide region of the second nucleic acid, wherein the at least one repetitive nucleotide region is defined as a) three more consecutively repeated G or C nucleotides and/or b) three or more consecutively repeated T nucleotides; wherein the recombinant poxvirus is stable through successive passaging.

[025] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned by practice of the invention. The objects and advantages of the invention will be

realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[026] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one or more embodiments of the invention and, together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[027] Figure 1 is a PCR analysis that illustrates the instability of the transgenes of PANVAC (MUC1 and CEA). Shown are the results of the PCR amplicon of the used site for integration of MUC1 and CEA within the TBC-FPV genome (IGR61/62). Highlighted is the height of the expected PCR fragment and a potential wt-fragment. Several deletion fragments of smaller size can be detected and are enriched during repeated passaging at either 34°C or 37°C. Results are shown for passage 0 to 7 of PANVA-F.

[028] Figures 2A and 2B depicts the amino acid sequences of MUC1 and the shuffling of the VNTR domain repeats according to various embodiments of the invention. A) The MUC1 amino acid as found in PANVAC (SEQ ID NO: 6). Illustrated are the 6 VNTRs found in the PANVAC MUC1. B) The MUC1 amino acid as found in mBN336, mBN373, and mBN420 (SEQ ID NO: 30). Illustrated are the 3VNTRs found in mBN336, mBN373, and mBN420 MUC1. Underlined amino acids represent amino acids modified to form the agonist epitopes of WO 2013/103658.

[029] Figures 3A-3C depict pairwise alignments and an exemplary codon optimization of the MUC1 VNTR domain repeats according to various embodiments of the invention. A) Alignment of the PANVAC VNTR #2 (SEQ ID NO: 7) and the mBN336, mBN373, mBN420 VNTR #1 (SEQ ID NO: 8), B) Alignment of the PANVAC VNTR #1 (SEQ ID NO: 9) and the mBN336, mBN373, mBN420 VNTR #2 (SEQ ID NO: 10), C) Alignment of the PANVAC VNTR #3 (SEQ ID NO: 11) and the mBN336, mBN373, mBN420 VNTR #3 (SEQ ID NO: 12). Underlined nucleotides represent nucleotide regions modified to form the agonist epitopes of WO 2013/103658.

[030] Figures 4A-4C depict pairwise alignments of the MUC1 coding sequences, as compared to PANVAC, used in the recombinant poxvirus based constructs in accordance with the present invention. A) MUC1 PANVAC (SEQ ID NO:1) versus MUC1 mBN336 (SEQ ID NO:2); B) MUC1 PANVAC (SEQ ID NO:1) versus MUC1 mBN373 (SEQ ID NO:3); C) MUC1 PANVAC (SEQ ID NO:1) versus MUC1 mBN420 (SEQ ID NO:5). Exemplary repetitive regions comprising one or more substitutions are underlined.

[031] Figure 5 depicts a pairwise alignment of the CEA coding sequence of mBN373 and mBN420 (SEQ ID NO: 14), as compared to CEA of PANVAC (SEQ ID NO: 13), used in the recombinant poxvirus based constructs in accordance with the present invention. Exemplary repetitive regions comprising one or more substitutions are underlined.

[032] Figure 6 depicts a pairwise alignment of the B7-1 coding sequence of mBN373 and mBN420 (SEQ ID NO: 15), as compared to B7-1 of PANVAC (SEQ ID NO:16), as compared to PANVAC, used in the recombinant poxvirus based constructs in accordance with the present invention. Exemplary repetitive regions are illustrated by the shown substitutions (non * regions of the alignment).

[033] Figure 7 depicts a pairwise alignment of an ICAM-1 coding sequence of mBN373 and mBN420 (SEQ ID NO: 18), as compared to PANVAC (SEQ ID NO:19), as compared to PANVAC, used in the recombinant poxvirus based constructs in accordance with the present invention. Exemplary repetitive regions are illustrated by the shown substitutions (non * regions of the alignment).

[034] Figure 8 depicts a pairwise alignment of an LFA-3 coding sequence of mBN373 and mBN420 (SEQ ID NO: 21), as compared to PANVAC (SEQ ID NO: 22), as compared to PANVAC, used in the recombinant poxvirus based constructs in accordance with the present invention. Exemplary repetitive regions are illustrated by the illustrated substitutions (non * regions of the alignment).

[035] Figure 9A and 9B illustrate experiments analyzing stability of a MUC1 transgene in mBN336. A) PCR results for stability of CEA over seven passages representative for passages during and beyond production of Clinical Trial Material (CTM)/ GMP material. B) PCR results for stability of MUC1 over seven passages representative for passages during and beyond production of CTM/ GMP material. C) PCR results for the stability of the TRICOM over 7 passages representative for passages during and beyond production of CTM/ GMP material. The recombination plasmids used for generation of MVA-mBN336B were used as positive controls, MVA-BN® was used as negative control (empty vector backbone) and H₂O was used as control for the PCR reaction.

[036] Figure 10A and 10B illustrate an analysis of Passages 5, 6, and 7 of mBN336. A) PCR amplification of Passage 7 samples sent for analysis by sequencing. Individual PCR amplifications were performed for each individual transgenes: CEA, MUC1, and TRICOM. B) Electropherograms of the MUC1 nt-sequence depicting the loci containing the detected point mutation leading to a frame shift originating in passage 5.

[037] Figure 11A and 11B illustrate experiments analyzing stability of a MUC1 transgene in mBN373. A) PCR analysis of the inserted transgenes for each passage. The recombination plasmid used for generation of FPV-mBN373B was used as positive control, FPV (strain TBC-FPV) was used as negative control. B) PCR analysis of FPV-mBN373B at passage seven resulted in the expected band size of 5566 bp (PCR1) and 5264 bp (PCR2) covering the inserted transgenes and each inserted flanking region. Sequence analysis confirmed genetic stability of the recombinant after 7 passages, being representative for passages during and beyond production of CTM/ GMP material.

[038] Figure 12 is a PCR analysis that analyzes the stability of the MUC1, CEA, and TRICOM transgenes in mBN420. Shown is the result of the PCR amplicon of the used site for integration of all five transgenes within the MVA-BN genome (IGR88/89). Highlighted is the height of the expected PCR fragment and a potential wt-fragment. Several deletion fragments of smaller size can be detected and are enriched during repeated passaging at 30°C. Results are shown for passage 0 to 7 of mBN420.

[039] All Pairwise alignments illustrated in the Figures were conducted using the Clustal Omega sequence Alignment tool, available at <http://www.ebi.ac.uk/Tools/msa/clustalo/>.

DETAILED DESCRIPTION OF THE INVENTION

[040] It is to be understood that both the foregoing Summary and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[041] PANVAC employs a heterologous prime-boost strategy using the recombinant poxviruses of vaccinia (PANVAC-V) and fowlpox (PANVAC-F), each expressing the transgenes MUC1, CEA, and TRICOM. PANVAC has been shown to be effective in treating cancer and is currently in clinical trials for various cancers, including colorectal cancer, ovarian cancer, breast cancer, and bladder cancer. MVA-CV301 is another heterologous vaccine combination undergoing clinical trials (*see, e.g.*, Gulley et al., Clin Cancer Res 2008; vol. 14:10, Tsang et al. Clin Cancer Res 2005; vol. 11). MVA-CV301 employs a heterologous prime-boost strategy using MVA and fowlpox each expressing the transgenes MUC1, CEA, and TRICOM.

[042] While PANVAC and MVA-CV301 are effective in treating cancer, the transgenes of the PANVAC recombinant poxviruses become less stable with successive passaging and production of the viruses. Shown in Tables 1 and 2, after successive passaging

of the PANVAC-V and PANVAC-F, the percentage of viruses expressing the MUC1 and CEA steadily decreases.

Table 1 Percent of Expressing Plaques in PANVAC-V MVB1, MVB2 and Passages

Protein	MVB	Mean Percentage of Expressing Plaques (%)				
		MVB	Passage 1*	Passage 2*	Passage 3*	Passage 4*
CEA	1	99.5	99.8	98.3	94.7	90.0
	2	100.0	97.6	95.1	91.8	89.0
MUC1	1	99.8	99.3	95.0	91.6	83.0
	2	99.7	98.2	95.9	86.3	73.6
B7.1	1	99.9	99.9	99.7	99.4	97.7
	2	99.9	100.0	99.9	99.8	99.1
ICAM-1	1	99.8	99.5	98.8	98.6	97.5
	2	99.6	99.4	99.1	98.2	98.2
LFA-3	1	100.0	99.9	99.7	99.5	98.5
	2	100.0	99.6	99.9	99.8	99.1

*Each number represents the mean values obtained from three independent passage experiments.

Table 2 Percent of Expressing Plaques in PANVAC-F MVB1, MVB2 and Passages

Protein	MVB	Mean Percentage of Expressing Plaques (%)				
		MVB	Passage 1*	Passage 2*	Passage 3*	Passage 4*
CEA	1	99.2	99.5	96.1	80.4	54.9
	2	100.0	99.4	98.8	89.8	63.9
MUC1	1	99.7	99.3	95.3	75.7	44.3
	2	99.6	99.8	98.3	89.4	55.4
B7.1	1	100.0	100.0	100.0	99.8	99.8
	2	99.5	99.2	99.7	100.0	99.5
ICAM-1	1	100.0	99.9	99.8	99.4	99.5
	2	99.8	99.5	99.7	100.0	99.9
LFA-3	1	100.0	100.0	100.0	100.0	99.9
	2	100.0	99.9	99.7	100.0	100.0

*Each number represents the mean values obtained from three independent passage experiments.

[043] In at least one aspect, the decrease in expression of MUC1 and/or CEA appears to be a result of an at least partial loss of the MUC1 and/or CEA transgenes. Figure 1 illustrates the loss of the MUC and CEA transgenic sequence of PANVAC. In Figure 1, Recombinant PANVAC-F product was expected to be at 4445 bp. However, as illustrated, experiments showed the presence of multiple lesser sized fragments, which were confirmed to be fragmented sequences of MUC1 and CEA (data not shown). The loss of expression and instability of the MUC1 transgene and in the previous recombinant poxviruses hinder the production and the purity of the CV301 recombinant poxviruses.

[044] Prior to creating the various nucleic acids and recombinant poxviruses of the present invention, in order to stabilize the transgenes, the inventors made multiple attempts to customize and/or modify the recombinant vaccinia, recombinant MVA, and recombinant fowlpox viruses of PANVAC, and MVA-CV301. Shown in Tables 3 and 4, modifications to the transgenes and/or the recombinant vaccinia, recombinant MVA, and recombinant fowlpox viruses included: (i) alternating or modifying which intergenic regions (IGRs) where transgenes were inserted, (ii) optimizing the codons of one or more transgenes, (iii) varying transgene promoters, and (iv) modifying the numbers and arrangements of VNTR regions in the MUC1 transgene. As described in the tables, many of the constructs failed to be stably generated due to either loss-of-function mutations or fragment deletions resulting in loss of transgene expression.

TABLE 3

	<u>Construct Attempts - MVA Virus</u>	
<u>Construct name</u>	<u>Construct details</u>	<u>Results</u>
MVA-mBN247	MUC/CEA/TRICOM in IGR148/149 Promoters & TGs exactly as in PANVAC-V	Generation failed
MVA-mBN269	CEA only (as in PANVAC-V) in IGR148/149	Stable
MVA-mBN317	CEA with optimized codon usage in IGR44/45 TRICOM unchanged in IGR148/149	Loss of CEA during generation already
MVA-mBN329	CEA (as in PANVAC-V) in IGR44/45 TRICOM unchanged in IGR148/149	Generation successful Stable expression of TGs for 7 passages at 30°C & 37°C (FACS by BN-CVD)
MVA-mBN332	MUC1-C3-opt6VNTRs in IGR88/89 CEA (as in PANVAC-V) in IGR44/45 TRICOM (as in PANVAC-V) in IGR148/149	Generation failed
MVA-mBN335	MUC1-C5-opt6VNTRs-SignMut in	Generation failed

	IGR88/89 CEA (as in PANVAC-V) in IGR44/45 TRICOM (as in PANVAC-V) in IGR148/149	
--	--	--

TABLE 4

	<u>Construct Attempts - Fowlpox Virus</u>	
<u>Construct name</u>	<u>Construct details</u>	<u>Results</u>
FPV-mBN285	CEA & TRICOM in BamJ (different to PANVAC-F) Promoters & TGs exactly as in PANVAC-F	Generation failed
FPV-mBN318	FPV-mBN285 + MUC1-C3-opt6VNTRs-SignMut in IGR61/62	Generation failed
FPV-mBN319	FPV-mBN285 + MUC1-C14-opt3VNTRs-SignMut in IGR61/62	Generation failed
FPV-mBN322	FPV-mBN285 + MUC1-C5-opt6VNTRs in IGR61/62	Generation failed
FPV-mBN338	FPV-mBN285 + MUC1-C5-opt6VNTRs-SignMut in IGR61/62	Generation failed
FPV-mBN339	FPV-mBN285 + MUC1-C13-opt3VNTRs in IGR61/62	Generation failed
FPV-mBN351	MUC1-C13-opt3VNTRs only in IGR61/62	Weak MUC-1 Expression
FPV-mBN352	MUC1/CEA/TRICOM in BamJ with FPV-40K promoter for MUC1-C13-opt3VNTRs	Single nucleotide mutations in CEA occurred repeatedly
FPV-mBN353	FPVmBN285 + (FPV-40K promoter)-MUC1-C13-opt3VNTRs with MUC1 in reverse orientation to ORFs of IGR61/62	Immediate loss of MUC1
FPV-mBN362	FPV-mBN351 & FPMBN285 co-infection (PrS)-MUC1-C13-opt3VNTRs in IGR61/62 & TRICOM in BamJ	Single nucleotide mutations in CEA occurred repeatedly

[045] After these multiple attempts, MVA-mBN336 was constructed. As described herein, MVA-mBN336 is an MVA-CV301 recombinant poxvirus including a modified MUC1, a CEA, and modified TRICOM transgenes. Shown in Figures 9 and 10, MVA-mBN336 demonstrated transgene stability as compared to PANVAC (see Figure 1 and table 1). Shown in Figure 10, the MVA-mBN336 showed stability of all of the transgenes (MUC1, CEA, and TRICOM, through Passage 4. Starting at Passage 5, a frameshift mutation was detected within a minor population of the analyzed material. The stability illustrated through passage 4 demonstrates the ability of the MVA-mBN336 to overcome the stability problems

associated with PANVAC and other attempts to generate a stable poxvirus including MUC1. The stability of MVA-mBN336 is additionally advantageous, as manufacture and larger scale production of MVA-based vaccines are typically taken from MVAs at passage 3 or passage 4. Thus, because MVA-mBN336 is stable through passage 4, large scale production can begin and significant regulatory hurdles with regard to stability can be overcome.

[046] To address and correct the instability problems, the nucleic acids of the present invention were synthesized and provide for one or more nucleic acids that encode for a MUC1 transgene, CEA transgene, and the TRICOM transgenes. As shown by the present disclosure, the MUC1, CEA, and the TRICOM nucleic acids of the present invention result in an improved genetic stability of the recombinant poxvirus and the transgenes included therein through successive passaging of the recombinant poxviruses.

[047] Thus, in various embodiments, the present invention provides a recombinant poxvirus having one or more novel nucleic acids that encode for MUC1, CEA, and/or TRICOM antigens. As provided in more detail herein, in at least one aspect, when incorporated as part of a recombinant poxvirus, the one or more modified MUC1, CEA, and/or TRICOM encoding nucleic acid sequences improve the stability and presence of transgenes in the recombinant poxvirus.

Definitions

[048] As used herein, the singular forms "a", "an", and "the", include plural references unless the context clearly indicates otherwise. Thus, for example, reference to "a nucleic acid" includes one or more of the nucleic acid and reference to "the method" includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

[049] Unless otherwise indicated, the term "at least" preceding a series of elements is to be understood to refer to every element in the series. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the present invention.

[050] Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step. When used herein the term "comprising" can be substituted with the term "containing" or "including" or sometimes when used herein with the term "having". Any of the

aforementioned terms (comprising, containing, including, having), though less preferred, whenever used herein in the context of an aspect or embodiment of the present invention can be substituted with the term "consisting of". When used herein "consisting of" excludes any element, step, or ingredient not specified in the claim element. When used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim.

[051] As used herein, the conjunctive term "and/or" between multiple recited elements is understood as encompassing both individual and combined options. For instance, where two elements are conjoined by "and/or", a first option refers to the applicability of the first element without the second. A second option refers to the applicability of the second element without the first. A third option refers to the applicability of the first and second elements together. Any one of these options is understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or" as used herein. Concurrent applicability of more than one of the options is also understood to fall within the meaning, and therefore satisfy the requirement of the term "and/or."

[052] "Mutation" described herein is as defined herein any a modification to a nucleic acid, such as deletions, additions, insertions, and/or substitutions.

[053] "Costimulatory molecules" as used herein, are molecules that, when bound to their ligand, deliver a second signal such that a T cell can become activated. The most well-known costimulatory molecule on the T cell is CD28, which binds to either B7-1 (also called B7.1 or CD80) or B7-2 (also known as CD86). An additional costimulatory molecule is B7-3. Accessory molecules that also provide a second signal for the activation of T cells include intracellular adhesion molecule (ICAM-1 and ICAM-2), leukocyte function associated antigen (LFA-1, LFA-2 and LFA-3). Integrins and tumor necrosis factor (TNF) superfamily members can also serve as co-stimulatory molecules.

[054] "Genetic stability", "stability", "Stability of expression", "stable through successive passaging", "stability through successive passaging" or "stability of expression through successive passaging" of the recombinant poxviruses when used herein in conjunction with the recombinant poxvirus, MUC1, CEA, TRICOM, and other transgenes is understood to mean that transgenic nucleotide sequences of the recombinant poxvirus remain materially intact and/or materially unchanged through successive passaging of the recombinant poxvirus until at least at passage 3 or passage 4. A recombinant poxvirus having stability at least through Passage 3 or passage 4 is particularly important as the final product generated by large scale manufacture and production of poxviruses are typically passage 3 or

passage 4. “Materially intact and/or materially unchanged” means the absence of single or fragment mutations (e.g., including substitutions, deletions, etc.) that cause a constant decrease of expression of the transgene as the number of passages increase. For example, shown in Tables 1 and 2, the expression levels of the various transgenes of PANVAC decreased as the number of passages increased. There is a variety of ways known in the art in which genetic stability or stability of transgenes can be analyzed, including, but not limited to, the assays described in Examples 2 through 4 of the instant application. Additional ways known in the art to measure stability include, but are not limited to, PCR, FACS, measurement of transgene co-expression by FACS, and so forth.

[055] A “host cell” as used herein is a cell that has been introduced with a foreign molecule, virus, or microorganism for the purpose of development and/or production of the foreign molecule, virus, or microorganism. In one non-limiting example, as described herein, a cell of a suitable cell culture as, e.g., CEF cells, can be infected with a poxvirus or, in other alternative embodiments, with a plasmid vector comprising a foreign or heterologous gene. Thus, the suitable cell cultures serve as a host to a poxvirus and/or foreign or heterologous gene.

[056] “Percent (%) sequence homology or identity” with respect to nucleic acid sequences described herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the reference sequence (i.e., the nucleic acid sequence from which it is derived), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent nucleotide sequence identity or homology can be achieved in various ways that are within the skill in the art, for example, using publically available computer software such as BLAST, ALIGN, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximum alignment over the full length of the sequences being compared.

[057] For example, an appropriate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, (1981), *Advances in Applied Mathematics* 2:482- 489. This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, *Atlas of Protein Sequences and Structure*, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov (1986), *Nucl. Acids Res.* 14(6):6745-6763. An exemplary implementation of this algorithm to determine percent identity of a sequence is

provided by the Genetics Computer Group (Madison, Wis.) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, Wis.). A preferred method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, Calif). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non- redundant, GenBank+EMBL+DDBJ+PDB+ GenBank CDS translations+Swiss protein+Spupdate+PIR. Details of these programs can be found at the following internet address: <http://blast.ncbi.nlm.nih.gov/>.

[058] The term "prime-boost vaccination" or "prime-boost regimen" refers to a vaccination strategy or regimen using a first priming injection of a vaccine targeting a specific antigen followed at intervals by one or more boosting injections of the same vaccine. Prime-boost vaccination may be homologous or heterologous. A homologous prime-boost vaccination uses a vaccine comprising the same antigen and vector for both the priming injection and the one or more boosting injections. A heterologous prime-boost vaccination uses a vaccine comprising the same antigen for both the priming injection and the one or more boosting injections but different vectors for the priming injection and the one or more boosting injections. For example, a homologous prime-boost vaccination may use a recombinant poxvirus comprising nucleic acids expressing one or more antigens for the priming injection and the same recombinant poxvirus expressing a one or more antigens for the one or more boosting injections. In contrast, a heterologous prime-boost vaccination may use a recombinant poxvirus comprising nucleic acids expressing one or more antigens for the priming injection and a different recombinant poxvirus expressing a one or more antigens for the one or more boosting injections.

[059] The term "recombinant" means a polynucleotide of semisynthetic, or synthetic origin which either does not occur in nature or is linked to another polynucleotide in an arrangement not found in nature.

[060] "Successive Passaging" as used herein, relates the production of recombinant viruses through the use of cell passaging. By way of example only, host cells are infected with a virus or recombinant virus in an initial passage. Viruses replicate and are produced in the initial passage. After infection and cultivation of host cells, viruses are harvested from the host cells and collected in a cell/viral suspension. This procedure is typically repeated multiple times in subsequent cell passages, each passage producing and replicating more recombinant viruses.

[061] As used herein, a "transgene" or "heterologous" gene is understood to be a nucleic acid or amino acid sequence which is not present in the wild-type poxviral genome (e.g., vaccinia, fowlpox, or MVA). The skilled person understands that a "transgene" or "heterologous gene", when present in a poxvirus, such as Vaccinia Virus, is to be incorporated into the poxviral genome in such a way that, following administration of the recombinant poxvirus to a host cell, it is expressed as the corresponding heterologous gene product, i.e., as the "heterologous antigen" and/or "heterologous protein." Expression is normally achieved by operatively linking the heterologous gene to regulatory elements that allow expression in the poxvirus-infected cell. Preferably, the regulatory elements include a natural or synthetic poxviral promoter.

[062] "TRICOM." Triad of COstimulatory Molecules (also known as TRICOM) includes B7-1 (also known as B7.1 or CD80), intracellular adhesion molecule-1 (ICAM-1, also known as CD54) and lymphocyte function-associated antigen-3 (LFA-3, also known as CD58), and commonly included in recombinant viral vectors (e.g., poxviral vectors) expressing a specific antigen in order to increase the antigen-specific immune response. The individual components of TRICOM can be under the control of the same or different promoters, and can be provided on the same vector with the specific antigen or on a separate vector. Exemplary vectors are disclosed, for example, in Hodge et al., "A Triad of Costimulatory Molecules Synergize to Amplify T-Cell Activation," *Cancer Res.* 59:5800-5807 (1999) and U.S. Patent No. 7,211,432 B2, both of which are incorporated herein by reference.

[063] A "vector" refers to a DNA or RNA plasmid or virus that can comprise a heterologous polynucleotide. The heterologous polynucleotide may comprise a sequence of interest for purposes of prevention or therapy, and may optionally be in the form of an

expression cassette. As used herein, a vector needs not be capable of replication in the ultimate target cell or subject. The term includes cloning vectors and viral vectors.

Novel MUC1 nucleic acid sequences

[064] With the development of the present invention, the inventors determined that over the course of passaging the recombinant poxviruses, in particular the orthopoxviruses (e.g., vaccinia virus, MVA, MVA-BN) and the avipoxviruses (e.g., fowlpox virus), one or more of the regions of the nucleic acids encoding for MUC1, CEA, and/or TRICOM became mutated (e.g., deleted, substituted, etc.), thereby contributing to the instability of the recombinant poxvirus and transgenes therein.

Modifications to the VNTR regions

[065] As noted previously, the VNTR region is an extracellular domain of MUC1 that includes a 20 amino acid variable number tandem repeat (VNTR) domain with the number of repeats varying from 20 to 120 in different individuals. See, Brayman et al. While the amino acid sequence of the VNTR domains typically are identical (see, e.g., Figure 2A), the nucleotide sequence of the VNTRs can vary. Shown in Figure 2A, as part of PANVAC, MUC1 was synthesized to have 6 VNTRs.

[066] In one aspect, over the course of the development of the present invention, it was determined that one or more modifications to the nucleic acids encoding the MUC1 VNTR region improved the stability of the MUC1 transgene in a recombinant poxvirus. More particularly, the inventors determined that shuffling the nucleic acids encoding the VNTRs further enhanced the stability of the MUC1 transgene as compared to PANVAC MUC1. As used herein “shuffling” the VNTRs is defined as rearranging the order of the nucleic acids encoding the VNTR domain repeats. Illustrated in Figures 2A and 2B is a non-limiting example of shuffling the VNTRs. In Figure 2A, the order of the PANVAC VNTR domains is shown as VNTR #s 1-6. Looking at Figure 2B, the nucleic acid encoding VNTR #1 of mBN336, mBN373, mBN420 corresponds with what is VNTR# in PANVAC. VNTR #2 of mBN336, mBN373, mBN420 corresponds with what is VNTR#1 in PANVAC. Thus, in synthesizing the MUC1 of mBN336, mBN373, mBN420, the order of PANVAC VNTRs #1 and 2 were shuffled.

[067] It is understood by the present invention that the VNTR domains shown in Figures 2A and 2B are merely representative of the MUC1 VNTR domains and that the numbers of VNTR domains and arrangements in which the VNTRs shuffled can vary.

[068] In addition to shuffling the VNTRs, it was determined that optimizing the codons of the VNTR domains further enhanced the stability of the MUC1 transgene. As used

herein, “optimizing the codons” is defined as substituting one or more nucleotides of the VNTRs in order to minimize the chance of mutations and/or deletions to the nucleotide sequences of the VNTRs due to the homology of the repetitive nucleotide sequences.

[069] In a more specific embodiment, depicted in the alignments of Figures 3A-3B, one or more of the nucleic acids of the present invention include various substitutions in the VNTR domains. Shown in Figure 3A, VNTR1 of mBN373 and mBN420 (hereinafter mBN373/420), comprises one or more nucleotide sequences encoding an agonist epitope from WO 2013/103658 (region indicated by underlining) in addition to the illustrated codon optimization substitutions. Shown in Figures 3B and 3C, VNTRs 2 and 3 of mBN373/420 modification comprise the illustrated codon optimization substitutions.

[070] It is understood by the present invention that the illustrated codon optimization modifications to VNTR domains shown in Figures 3A-3C are merely representative of the MUC1 VNTR domain codon optimizations. By way of example only, it is contemplated by the present invention that alternative nucleotides may be substituted at the particular points of modification in the VNTR domains. It is additionally contemplated that the particular points of the modification in the VNTR domains may vary such that the modification is a silent modification. A silent modification, as used herein, means that the modification does not affect the amino acid sequence of the MUC1 antigen.

[071] Thus, in one embodiment, the MUC1 nucleic acids of the present invention comprise one or more VNTR domain regions that are 1) shuffled and 2) codon optimized. Modifications to non-VNTR regions of MUC1

[072] In another aspect of the present invention, one more modifications were made to those regions outside of the VNTR domains. In a more specific aspect, over the course of the development of the present invention, it was determined that one or more modifications in those nucleotide regions outside of the MUC1 VNTR region (non-VNTR regions) improved the stability of the MUC1 transgene. A representative sample of those regions (underlined nucleotides) is illustrated below. The VNTR region is shaded gray.

ATGACACCGG	<u>GCACCCAGTC</u>	<u>TCCTTTCTTC</u>	<u>CTGCTGCTGC</u>	<u>TCCTCACAGT</u>	GCTTACAGTT	60
GTTA <u>CGGGTT</u>	CTGGTCATGC	AAGCTCTACC	CCAGGTGGAG	AAAAGGAGAC	TTCGGCTACC	120
<u>CAGAGAAGTT</u>	CAGTGCCCAG	CTCTACTGAG	AAGAATGCTG	TGAGTATGAC	AAGCTCCGTA	180
CTCTCCAGCC	<u>ACAGCCCCGG</u>	<u>TTCAGGCTCC</u>	<u>TCCACCACTC</u>	<u>AGGGACAGGA</u>	<u>TGTCACCTCT</u>	240
<u>GCCCCGGCCA</u>	<u>CGGAACCAGC</u>	<u>TTCAGGTTC</u>	<u>GCTGCCTTGT</u>	<u>GGGGACAGGA</u>	<u>TGTCACCTCG</u>	300
GTACCAGTTA	CTAGACCAGC	TTTAGGTAGC	ACAGCACCTC	CTGCTCATGG	AGTAACTAGT	360
GCTCCTGATA	CTCGTCCAGC	TCCTGGCAGT	ACTGCACCAC	CGGCACATGG	CGTAACATCA	420
GCACCTGATA	CAAGACCTGC	ACCTGGATCT	ACAGCGCCGC	CTGCGCACGG	AGTGACATCG	480
GCGCCCGATA	CGCGCCCCGC	TCCCGGTAGC	ACCGCACCGC	CCGCCACGG	TGTTACAAGT	540

GCACCCGATA	CCCGGCCGGC	ACCCGGAAGT	ACCGCTCCAC	CTGCACACGG	GGTCACAAGC	600
GCGCCAGACA	CTCGACCTGC	GCCAGGGTOG	ACTGCCCTC	CGGCGCATGG	TGTGACCTCA	660
GCTCCTGACA	CAAGGCCAGC	CCCAGCTAGC	ACTCTGGTGC	ACAACGGCAC	CTCTGCCAGG	720
GCTACCACAA	CCCCAGCCAG	CAAGAGCACT	CCATTCTCAA	<u>TTCCCAGCCA</u>	<u>CCACTCTGAT</u>	780
ACTCCTACCA	<u>CCCTTGCCAG</u>	CCATAGCACC	AAGACTGATG	CCAGTAGCAC	TCACCATAGC	840
ACGGTACCTC	CTCTCACCTC	CTCCAATCAC	AGCACTTCTC	CCCAGTTGTC	TACTGGGGTC	900
<u>TCTTTCTTTT</u>	<u>TCCTGTCTTT</u>	<u>TCACATTTC</u>	<u>AACCTCCAGT</u>	<u>TTAATTCTCT</u>	<u>TCTGGAAGAT</u>	960
CCCAGCACCG	ACTACTACCA	AGAGCTGCAG	AGAGACATTT	<u>CTGAAATGTT</u>	<u>TTTGCAGATT</u>	1020
TATAACAAG	<u>GGGGTTTTCT</u>	<u>GGGCCTCTCC</u>	AATATTAAGT	TCAGGCCAGG	ATCTGTGGTG	1080
<u>GTACAATTGA</u>	<u>CTCTGGCCTT</u>	<u>CCGAGAAGGT</u>	ACCATCAATG	TCCACGACGT	GGAGACACAG	1140
TTCAATCAGT	<u>ATAAAACGGA</u>	<u>AGCAGCCTCT</u>	<u>CGATATAACC</u>	TGACGATCTC	AGACGTCAGC	1200
GTGAGTGATG	TGCCATTTCC	TTTCTCTGCC	CAGTCTGGGG	CTGGGGTGCC	AGGCTGGGGC	1260
<u>ATCGCGCTGC</u>	<u>TGGTGTCTGT</u>	<u>CTGTGTTCTG</u>	<u>GTTGCGCTGG</u>	<u>CCATTGTCTA</u>	<u>TCTCATTGCC</u>	1320
TTGGCTGTCT	<u>GTCAGTGCCG</u>	<u>CCGAAAGAAC</u>	TACGGGCAGC	TGGACATCTT	<u>TCCAGCCCCG</u>	1380
GATACCTACC	ATCCTATGAG	CGAGTACCCC	ACCTACCACA	CCCATGGGCG	CTATGTGCCC	1440
CCTAGCAGTA	CCGATCGTAG	CCCCTATGAG	<u>AAGTTTCTG</u>	<u>CAGGTAATGG</u>	<u>TGGCAGCAGC</u>	1500
CTCTCTTACA	CAAACCCAGC	AGTGGCAGCC	ACTTCTGCCA	ACTTGTAG		1548

[SEQ ID NO:1]

[073] To generate a recombinant poxvirus which is stable through successive passaging of the virus, the one or more nucleic acids of the present invention were synthesized. More particularly, illustrated in Figures 2A through 2C, one or more substitutions were made to one or more of the underlined areas outside of the VNTR regions of the PANVAC MUC1 (SEQ ID NO:1), as shown.

[074] Thus, in one embodiment of the invention, there is a novel MUC1nucleic acid that comprises a substitution to at least one of the repetitive nucleotide regions outside of the VNTR regions of the MUC1 nucleic acid. In at least one aspect, one or more of the repetitive regions are defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides. In more specific aspects, one or more of repetitive nucleotide regions is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides. In certain other more specific aspects, the consecutively repeated nucleotides are defined as (i) consecutive G nucleotides, (ii) consecutive C nucleotides, and/or (iii) consecutive T nucleotides.

[075] As shown by Figures 2A through 2C, the novel MUC1nucleic acid can comprise a substitution in at least 2, 3, 4, or 5 repetitive nucleotide regions outside of the VNTR regions of the MUC1 nucleic acid. In further aspects, the novel MUC1nucleic acid can comprise a substitution in at least 10, 15, 20, or 25 repetitive nucleotide regions outside of the VNTR regions.

[076] In still additional aspects, the novel MUC1nucleic acid can comprise at least one substitution in those regions outside of the VNTR regions that are more prone to mutate

over successive passaging of the recombinant poxvirus. In an exemplary aspect, the novel MUC1 nucleic acid can comprise at least one substitution in one or more of those MUC1 nucleotide repetitive regions outside of the VNTR regions selected from nucleotide regions and/or combinations thereof of PANVAC MUC1 (SEQ ID NO:1) shown in Table 5.

TABLE 5

7-16	19-32	40-45	65-68	122-128	136-138
194-200	207-213	222-224	240-253	296-299	705-714
731-734	761-765	770-773	791-795	847-864	880-883
895-922	933-953	1004-1006	1009-1113	1030-1050	1075-1081
1085-1090	1097-1102	1153-1156	1166-1171	1201-1212	1237-1246
1264-1280	1294-1300	1328-1332	1335-1346	1353-1357	1375-1381
1407-1410	1418-1423	1426-1431	1437-1442	1449-1454	1459-1464
1471-1479	1494-1500				

More preferably, the novel MUC1 nucleic acid can comprise at least one substitution in those MUC1 nucleotide repetitive regions outside of the VNTR regions selected from nucleotide regions and/or combinations thereof of PANVAC MUC1 (SEQ ID NO:1) shown in Table 6.

TABLE 6

7-16	19-32	40-45	65-68	122-128	136-138
194-200	207-213	222-224	240-253	296-299	705-708
710-714	731-734	761-765	770-773	791-795	847-855
857-864	880-883	895-898	899-914	916-922	933-937
940-943	945-953	1004-1006	1009-1113	1030-1050	1075-1081
1085-1090	1097-1102	1153-1156	1166-1171	1201-1212	1237-1240
1243-1246	1264-1280	1294-1300	1328-1332	1335-1337	1338-1343
1344-1346	1353-1357	1375-1381	1407-1410	1418-1423	1426-1431

1437-1442	1449-1454	1459-1464	1471-1479	1494-1500	
-----------	-----------	-----------	-----------	-----------	--

It is understood by the present invention that the nucleotide positions listed in Tables 5 and 6 are merely representative of the MUC1 nucleic acid repetitive regions found in the non-VNTR regions of MUC1. Thus, while a repetitive region described herein has a specified nucleotide position in SEQ ID NO:1 (e.g., 240-253), that particular region may correspond to another nucleotide position in another MUC1 nucleic acid.

[077] In additional embodiments, the modifications to the repetitive regions outside of the VNTRs, and/or the modifications in the VNTR regions is a silent modification, meaning that the modification does not affect the amino acid sequence of the MUC1 antigen. In at least one aspect, enhancing the stability of the MUC1 transgene by modifying one or more repetitive regions was challenging in that only certain nucleotides and/or repetitive regions could be modified without affecting the amino acid sequence of the MUC1.

[078] In view of the foregoing, in one or more embodiments, the present invention includes one or more MUC1 nucleic acids comprising 1) one or more modifications to the VNTR domain repeats selected from a) shuffling and b) codon optimization; and 2) one or modifications to repetitive regions outside of the VNTRs.

[079] In another aspect, the MUC1 nucleic acids of the present invention can include one or more modifications configured to enhance the immunogenicity of the MUC1 transgene in a subject. In one non-limiting example, the MUC1 nucleic acids can be modified to include one or more of the agonist epitopes described in WO 2013/103658, which is incorporated by reference herein. In a more specific embodiment, the MUC1 nucleic acids of the present invention include agonist epitopes selected from the group consisting of: YLAPPAHGV [SEQ ID NO: 24], YLDTRPAPV [SEQ ID NO: 25], YLAIVYLIAl [SEQ ID NO: 26], YLIALAVCQV [SEQ ID NO: 27], YLSYTNPAV [SEQ ID NO: 28], and SLFRSPYEK [SEQ ID NO: 29] (underlined portions are substituted amino acids).

[080] In preferred embodiments, the MUC1 nucleic acid comprises a nucleotide sequence at least 95% homologous to SEQ ID NO: 2 (336 MUC), SEQ ID NO: 3 (373 MUC), SEQ ID NO: 4 (399/400 MUC1), or SEQ ID NO: 5 (420 MUC1). In still additional preferred embodiments, the MUC1 nucleic acid comprises a nucleotide sequence at least 96%, 97%, or 98% homologous to SEQ ID NO: 2 (336 MUC), SEQ ID NO:3 (373 MUC), SEQ ID NO: 4 (399/400 MUC1), or SEQ ID NO: 5 (420 MUC1). In a more preferred

embodiment, the MUC1 nucleic acid comprises a nucleotide sequence selected from SEQ ID NO: 2 (336 MUC), SEQ ID NO:3 (373 MUC), SEQ ID NO: 4 (399/400 MUC1), or SEQ ID NO: 5 (420 MUC1).

[081] In still other preferred embodiments, the MUC1 nucleic acid comprises a nucleotide sequence at least 95% homologous to SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, or SEQ ID NO: 34. In still additional preferred embodiments, the MUC1 nucleic acid comprises a nucleotide sequence at least 96%, 97%, or 98% homologous to SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, or SEQ ID NO: 34. In a more preferred embodiment, the MUC1 nucleic acid comprises a nucleotide sequence selected from SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, or SEQ ID NO: 34.

Novel CEA nucleic acid sequences

[082] In another aspect of the present invention, it was determined that one or more modifications in the repetitive regions of the CEA nucleic acids improved the stability of the CEA transgene. A representative sample of those regions is illustrated in the pairwise alignment of Figure 5. Those exemplary repetitive regions are illustrated by the shown substitutions (non * regions of the alignment).

[083] In at least one aspect, the substitution further enhanced the stability of a recombinant poxvirus. This is demonstrated at least in part by the stability data for mBN336 shown in Figures 9 and 10. As previously noted, mBN336 includes MUC1, CEA, and TRICOM. While mBN336 includes a modified MUC1 nucleic acid, mBN336 does not include any additional modifications to CEA, and include only intermediate modifications to the TRICOM costimulatory molecules. Thus, while the modifications to MUC1 disclosed herein improved the stability of a recombinant poxvirus, instability starting at passage 5 remained. Once the modified CEA was included as part of the fowlpox virus in mBN373, stability of the transgene and the fowlpox virus was demonstrated past passage 5 and into passage 7. (see, e.g., Figure 11).

[084] Accordingly, in various embodiments, the present invention includes a nucleic acid encoding a CEA peptide (CEA nucleic acid) comprising at least one nucleotide substitution in at least one repetitive nucleotide region of the CEA nucleic acid, wherein the at least one repetitive nucleotide region is defined as a) three more consecutively repeated G or C nucleotides and/or b) three or more consecutively repeated T nucleotides. In additional embodiments, the repetitive nucleotide regions are further defined as a) three or more consecutively repeated G nucleotides and/or b) three or more consecutively repeated C nucleotides.

[085] In preferred embodiments, the repetitive nucleotide regions of the CEA nucleic acid are defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T nucleotides. In additional preferred embodiments, the repetitive nucleotide region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

[086] In one or more embodiments, the CEA nucleic acid includes at least one substitution to at least 2, 3, 4, 5, or 10 of the repetitive nucleotide regions of the second nucleic acid. In a preferred embodiment, the CEA nucleic acid comprises at least one nucleotide substitution in at least 10, at least 12, at least 15, and/or at least 19 repetitive nucleotide regions. In a more preferred embodiment, the CEA nucleic acid comprises at least one nucleotide substitution in 19 regions of the second nucleic acid.

[087] In more preferred embodiments, the CEA nucleic acid comprises SEQ ID NO: 14 (mBN373/420 CEA).

Novel TRICOM nucleic acid sequences

[088] In another aspect of the present invention, one more modifications were made to one or more nucleic acids encoding the TRICOM costimulatory molecules. In a more specific aspect, over the course of the development of the present invention, it was determined that one or more modifications in the repetitive regions of the TRICOM nucleic acids improved the stability of the TRICOM transgenes. A representative sample of those regions is illustrated in the pairwise alignment of Figure 6-8. Those exemplary repetitive regions are illustrated by the shown substitutions (non * regions of the alignment).

[089] In at least one aspect, the one or more substitutions further enhanced the stability of a recombinant poxvirus. This is demonstrated at least in part by the stability data for mBN336 shown in Figures 9 and 10. As previously noted, mBN336 includes a modified MUC1. mBN336, however, does not include any additional modifications to CEA, and includes only intermediate modifications to the TRICOM costimulatory molecules. Thus, while the modifications to MUC1 disclosed herein improved the stability of mBN336, instability past passage 5 remained. Once the modified transgenes were included as part of the fowlpox virus, stability of the transgene and poxvirus was demonstrated past passage 5 and into passage 7. (see, e.g., Figure 11).

[090] In one embodiment, the novel TRICOM costimulatory molecules comprise a nucleotide sequence at least 80% homologous to SEQ ID NO: 15 or 17 (for B7-1), a nucleotide sequence at least 80 % homologous to SEQ ID NO: 18 or 20 (for ICAM-1), and a

nucleotide sequence at least 80% homologous to SEQ ID NO: 21 or 23 (for LFA-3). In still additional preferred embodiments, the TRICOM nucleic acids comprises a nucleotide sequence at least 85%, 90%, or 95% homologous to SEQ ID NO:15 or 17 (for B7-1), SEQ ID NO: 18 or 20 (for ICAM-1), and/or SEQ ID NO: 21 or 23 (for LFA-3). In still more preferred embodiments, the TRICOM nucleic acids comprises a nucleotide sequence at least 85%, 90%, or 95% homologous to SEQ ID NO:17 (for B7-1), SEQ ID NO: 20 (for ICAM-1), and/or SEQ ID NO: 23 (for LFA-3)

[091] In another embodiment, the TRICOM costimulatory molecules comprise SEQ ID NO: 15 or 17 (for B7-1), SEQ ID NO: 18 or 20 (for ICAM-1), and/or SEQ ID NO: 21 or 23 (for LFA-3).

[092] In yet another embodiment, the TRICOM costimulatory molecules comprise SEQ ID NO: 17 (for B7-1), SEQ ID NO: 20 (for ICAM-1), and/or SEQ ID NO: 23 (for LFA-3).

[093] In one preferred embodiment, the novel TRICOM costimulatory molecules comprise a nucleotide sequence at least 80% homologous to SEQ ID NO: 15 (for B7-1), a nucleotide sequence at least 80 % homologous to SEQ ID NO: 18 (for ICAM-1), and a nucleotide sequence at least 80% homologous to SEQ ID NO: 21 (for LFA-3). In still additional preferred embodiments, the TRICOM nucleic acids comprises a nucleotide sequence at least 85%, 90%, or 95% homologous to SEQ ID NO:15 (for B7-1), SEQ ID NO: 18 (for ICAM-1), and/or SEQ ID NO: 21 (for LFA-3).

[094] In another preferred embodiment, the novel TRICOM costimulatory molecules comprise a nucleotide sequence at least 80%, 90%, or 95% homologous to SEQ ID NO: 17 (for B7-1), a nucleotide sequence at least 80 %, 90%, or 95% homologous to SEQ ID NO: 20 (for ICAM-1), and a nucleotide sequence at least 80%, 90%, or 95% homologous to SEQ ID NO: 23 (for LFA-3).

[095] In another embodiment, the TRICOM costimulatory molecules comprise SEQ ID NO: 15 (for B7-1), SEQ ID NO: 18 (for ICAM-1), and/or SEQ ID NO: 21 (for LFA-3).

[096] In another embodiment, the TRICOM costimulatory molecules comprise SEQ ID NO: 17 (for B7-1), SEQ ID NO: 20 (for ICAM-1), and/or SEQ ID NO: 23 (for LFA-3).

[097] It is contemplated that the present disclosure embodies those nucleic acid sequences that are complementary to the novel nucleic acid sequences provided herein.

Recombinant Poxviruses

[098] In one or more embodiments, the invention includes a recombinant poxvirus comprising one or more of the MUC1 nucleic acids described herein. In more preferred

embodiments, the recombinant poxvirus comprises a MUC1 nucleic acid sequence and a CEA nucleic acid sequence described herein.

[0099] In preferred embodiments, the MUC1 nucleic acid comprises a nucleotide sequence at least 95% homologous to SEQ ID NO:2, SEQ ID NO: 3 (373 MUC), SEQ ID NO: 5 (420 MUC1), or SEQ ID NO: 4 (399/400 MUC1), and a CEA nucleic acid sequence comprising SEQ ID NO: 13 or 14.

[0100] In still additional embodiments, the recombinant poxviruses of the present disclosure include one or more costimulatory molecules, such as but not limited to, those described herein. In one preferred embodiment, the costimulatory molecules include TRICOM (B7-1, ICAM-1, and LFA-3). In a more preferred embodiment, the B7-1 costimulatory molecules are selected from a nucleic acid sequence comprising SEQ ID NO: 15, SEQ ID NO: 16, and SEQ ID NO: 17. In a more preferred embodiment, the ICAM-1 costimulatory molecule is selected from a nucleic acid sequence comprising SEQ ID NO:18, SEQ ID NO: 19, and SEQ ID NO: 20. In a more preferred embodiment, the LFA-3 costimulatory molecule is selected from a nucleic acid sequence comprising SEQ ID NO: 21, SEQ ID NO: 22, and SEQ ID NO: 23. In a more preferred embodiment, the B7-1, ICAM-1, and LFA-3 are selected from a nucleic acid sequence comprising SEQ ID NO: 15, SEQ ID NO: 18, and SEQ ID NO: 21, respectively. In another more preferred embodiment, the B7-1, ICAM-1, and LFA-3 are selected from a nucleic acid sequence comprising SEQ ID NO: 17, SEQ ID NO: 20, and SEQ ID NO: 23, respectively

[0101] In the various embodiments of the present disclosure, the recombinant poxvirus is preferably an orthopoxvirus such as, but not limited to, a vaccinia virus, a Modified Vaccinia Ankara (MVA) virus, MVA-BN, or derivatives of MVA-BN.

[0102] Examples of vaccinia virus strains are the strains Temple of Heaven, Copenhagen, Paris, Budapest, Dairen, Gam, MRIVP, Per, Tashkent, TBK, Tom, Bern, Patwadangar, BIEM, B-15, Lister, EM-63, New York City Board of Health, Elstree, Ikeda and WR. A preferred vaccinia virus (VV) strain is the Wyeth (DRYVAX) strain (U.S. Patent 7,410,644).

[0103] Another preferred VV strain is a modified vaccinia virus Ankara (MVA) (Sutter, G. et al. [1994], Vaccine 12: 1032-40). Examples of MVA virus strains that are useful in the practice of the present invention and that have been deposited in compliance with the requirements of the Budapest Treaty are strains MVA 572, deposited at the European Collection of Animal Cell Cultures (ECACC), Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and

Research, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom, with the deposition number ECACC 94012707 on January 27, 1994, and MVA 575, deposited under ECACC 00120707 on December 7, 2000, MVA-BN, deposited on Aug. 30, 2000 at the European Collection of Cell Cultures (ECACC) under number V00083008, and derivatives of MVA-BN, are additional exemplary strains.

[0104] “Derivatives” of MVA-BN refer to viruses exhibiting essentially the same replication characteristics as MVA-BN, as described herein, but exhibiting differences in one or more parts of their genomes. MVA-BN, as well as derivatives thereof, are replication incompetent, meaning a failure to reproductively replicate *in vivo* and *in vitro*. More specifically *in vitro*, MVA-BN or derivatives thereof have been described as being capable of reproductive replication in chicken embryo fibroblasts (CEF), but not capable of reproductive replication in the human keratinocyte cell line HaCat (Boukamp et al (1988), J. Cell Biol. 106:761-771), the human bone osteosarcoma cell line 143B (ECACC Deposit No. 91112502), the human embryo kidney cell line 293 (ECACC Deposit No. 85120602), and the human cervix adenocarcinoma cell line HeLa (ATCC Deposit No. CCL-2). Additionally, MVA-BN or derivatives thereof have a virus amplification ratio at least two fold less, more preferably three-fold less than MVA-575 in Hela cells and HaCaT cell lines. Tests and assay for these properties of MVA-BN and derivatives thereof are described in WO 02/42480 (U.S. Patent application No. 2003/0206926) and WO 03/048184 (U.S. Patent application No. 2006/0159699).

[0105] The term “not capable of reproductive replication” or “no capability of reproductive replication” in human cell lines *in vitro* as described in the previous paragraphs is, for example, described in WO 02/42480, which also teaches how to obtain MVA having the desired properties as mentioned above. The term applies to a virus that has a virus amplification ratio *in vitro* at 4 days after infection of less than 1 using the assays described in WO 02/42480 or in U.S. Patent No. 6,761,893.

[0106] The term “failure to reproductively replicate” refers to a virus that has a virus amplification ratio in human cell lines *in vitro* as described in the previous paragraphs at 4 days after infection of less than 1. Assays described in WO 02/42480 or in U.S. Patent No. 6,761,893 are applicable for the determination of the virus amplification ratio.

[0107] The amplification or replication of a virus in human cell lines *in vitro* as described in the previous paragraphs is normally expressed as the ratio of virus produced from an infected cell (output) to the amount originally used to infect the cell in the first place (input) referred to as the “amplification ratio”. An amplification ratio of “1” defines an

amplification status where the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells, meaning that the infected cells are permissive for virus infection and reproduction. In contrast, an amplification ratio of less than 1, i.e., a decrease in output compared to the input level, indicates a lack of reproductive replication and therefore attenuation of the virus.

[0108] In another embodiment, the recombinant poxvirus including the MUC1 and/or other nucleic acids disclosed herein is an avipoxvirus, such as but not limited to, a fowlpox virus.

[0109] The term "avipoxvirus" refers to any avipoxvirus, such as Fowlpoxvirus, Canarypoxvirus, Uncopoxvirus, Mynahpoxvirus, Pigeonpoxvirus, Psittacinepoxvirus, Quailpoxvirus, Peacockpoxvirus, Penguinpoxvirus, Sparrowpoxvirus, Starlingpoxvirus and Turkeypoxvirus. Preferred avipoxviruses are Canarypoxvirus and Fowlpoxvirus.

[0110] Examples of a fowlpox virus are strains FP-1, FP-5, TROVAC (U.S. Pat. No. 5,766,598), POXVAC-TC (U.S. Patent 7,410,644), TBC-FPV (Therion Biologics- FPV), FP-1 is a Duvette strain modified to be used as a vaccine in one-day old chickens. The strain is a commercial fowlpox virus vaccine strain designated O DCEP 25/CEP67/2309 October 1980 and is available from Institute Merieux, Inc. FP-5 is a commercial fowlpox virus vaccine strain of chicken embryo origin available from American Scientific Laboratories (Division of Schering Corp.) Madison, Wis., United States Veterinary License No. 165, serial No. 30321.

[0111] In certain preferred embodiments, there is a recombinant orthopoxvirus, such as Vaccinia, MVA, MVA-BN, or derivatives of MVA-BN comprising a MUC1 nucleic acid sequence selected from SEQ ID NO: 5 (420 MUC1), SEQ ID NO: 4 (399/400 MUC1), SEQ ID NO:3 (373 MUC1), OR SEQ ID NO:2 (336 MUC1). In certain more preferred embodiments, the recombinant orthopoxvirus is an MVA virus comprising a MUC1 nucleic acid sequence selected from SEQ ID NO: 2 (420 MUC1), a CEA nucleic acid selected from SEQ ID NO: 13 or 14, and TRICOM. In a most preferred embodiment, there is a recombinant MVA comprising a MUC1 nucleic acid sequence comprising SEQ ID NO: 2 (336 MUC1), a CEA nucleic acid comprising SEQ ID NO: 13, and TRICOM. In another most preferred the TRICOM includes one or more nucleic acids comprising SEQ ID NO: 17, (B7-1), SEQ I NO: 20 (ICAM-1), and SEQ ID NO: 23 (LFA-3).

[0112] In certain other preferred embodiments, there is a recombinant avipoxvirus, such as a fowlpox virus, comprising a MUC1 nucleic acid sequence comprising SEQ ID NO: 3 (373 MUC1). In certain more preferred embodiments, the recombinant avipoxvirus is a fowlpox virus comprising a MUC1 nucleic acid comprising SEQ ID NO: 3 (373), a CEA

nucleic acid selected from SEQ ID NO: 13 or 14, and TRICOM. In a most preferred embodiment, there is a recombinant fowlpox virus comprising a MUC1 nucleic acid sequence comprising SEQ ID NO: 3 (373 MUC1), a CEA nucleic acid comprising SEQ ID NO: 14, and TRICOM. In another most preferred the TRICOM includes one or more nucleic acids comprising SEQ ID NO: 15 (B7-1), SEQ I NO: 18 (ICAM-1), and SEQ ID NO: 21(LFA-3).

Expression Cassettes/Control Sequences

[0113] In various aspects, the one or more nucleic acids described herein are embodied in in one or more expression cassettes in which the one or more nucleic acids are operatively linked to expression control sequences. “Operably linked” means that the components described are in relationship permitting them to function in their intended manner e.g., a promoter to transcribe the nucleic acid to be expressed. An expression control sequence operatively linked to a coding sequence is joined such that expression of the coding sequence is achieved under conditions compatible with the expression control sequences. The expression control sequences include, but are not limited to, appropriate promoters, enhancers, transcription terminators, a start codon at the beginning a protein-encoding open reading frame, splicing signals for introns, and in-frame stop codons. Suitable promoters include, but are not limited to, the SV40 early promoter, an RSV promoter, the retrovirus LTR, the adenovirus major late promoter, the human CMV immediate early I promoter, and various poxvirus promoters including, but not limited to the following vaccinia virus or MVA-derived and FPV-derived promoters: the 30K promoter, the I3 promoter, the PrS promoter, the PrS5E promoter, the Pr7.5K, the Pr13.5 long promoter, the 40K promoter, the MVA-40K promoter, the FPV 40K promoter, 30k promoter, the PrSynIIIm promoter, and the PrLE1 promoter. Additional promoters are further described in WO 2010/060632, WO 2010/102822, WO 2013/189611 and WO 2014/063832 which are incorporated fully by reference herein.

[0114] Additional expression control sequences include, but are not limited to, leader sequences, termination codons, polyadenylation signals and any other sequences necessary for the appropriate transcription and subsequent translation of the nucleic acid sequence encoding the desired recombinant protein (e.g., MUC1, CEA, and/or TRICOM) in the desired host system. The poxvirus vector may also contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the desired host system. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1 987)

in "Current Protocols in Molecular Biology," John Wiley and Sons, New York, N.Y.) and are commercially available.

In certain embodiments, the recombinant orthopoxvirus and/or avipoxvirus of the present disclosure comprises one or more cytokines, such as IL-2, IL-6, IL-12, RANTES, GM-CSF, TNF- α , or IFN- γ , one or more growth factors, such as GM-CSF or G-CSF, one or more costimulatory molecules, such as ICAM-1, LFA-3, CD72, B7-1, B7-2, or other B7 related molecules; one or more molecules such as OX-40L or 41 BBL, or combinations of these molecules, can be used as biological adjuvants (see, for example, Salgaller et al., 1998, J. Surg. Oncol. 68(2):122-38; Lotze et al., 2000, Cancer J. Sci. Am. 6(Suppl 1):S61-6; Cao et al., 1998, Stem Cells 16(Suppl 1):251-60; Kuiper et al., 2000, Adv. Exp. Med. Biol. 465:381-90). These molecules can be administered systemically (or locally) to the host. In several examples, IL-2, RANTES, GM-CSF, TNF- α , IFN- γ , G-CSF, LFA-3, CD72, B7-1, B7-2, B7-1 B7-2, OX-40L, 41 BBL and ICAM-1 are administered.

Generation of Recombinant Poxviruses comprising Transgenes

[0115] The recombinant poxviruses provided herein can be generated by routine methods known in the art. Methods to obtain recombinant poxviruses or to insert exogenous coding sequences into a poxviral genome are well known to the person skilled in the art. For example, methods for standard molecular biology techniques such as cloning of DNA, DNA and RNA isolation, Western blot analysis, RT-PCR and PCR amplification techniques are described in Molecular Cloning, A laboratory Manual (2nd Ed.) [J. Sambrook et al., Cold Spring Harbor Laboratory Press (1989)], and techniques for the handling and manipulation of viruses are described in Virology Methods Manual [B.W.J. Mahy et al. (eds.), Academic Press (1996)]. Similarly, techniques and know-how for the handling, manipulation and genetic engineering of MVA are described in Molecular Virology: A Practical Approach [A.J. Davison & R.M. Elliott (Eds.), The Practical Approach Series, IRL Press at Oxford University Press, Oxford, UK (1993)(see, e.g., Chapter 9: Expression of genes by Vaccinia virus vectors)] and Current Protocols in Molecular Biology [John Wiley & Son, Inc. (1998)(see, e.g., Chapter 16, Section IV: Expression of proteins in mammalian cells using vaccinia viral vector)].

[0116] For the generation of the various recombinant poxviruses disclosed herein, different methods may be applicable. The DNA sequence to be inserted into the virus can be placed into an E. coli plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA sequence to be inserted can be ligated to a promoter. The promoter-gene linkage can be positioned in the plasmid construct so that the

promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of poxviral DNA containing a non-essential locus. The resulting plasmid construct can be amplified by propagation within *E. coli* bacteria and isolated. The isolated plasmid containing the DNA gene sequence to be inserted can be transfected into a cell culture, e.g., of chicken embryo fibroblasts (CEFs), at the same time the culture is infected with poxvirus. Recombination between homologous poxviral DNA in the plasmid and the viral genome, respectively, can generate a poxvirus modified by the presence of foreign DNA sequences.

[0117] According to a preferred embodiment, a cell of a suitable cell culture as, e.g., CEF cells, can be infected with a poxvirus. The infected cell can be, subsequently, transfected with a first plasmid comprising a foreign or heterologous gene or genes, such as one or more of the MUC1, CEA, and/or TRICOM nucleic acids provided in the present disclosure; preferably under the transcriptional control of a poxvirus expression control element. As explained above, the plasmid also comprises sequences capable of directing the insertion of the exogenous sequence into a selected part of the poxviral genome. Optionally, the plasmid vector also contains a cassette comprising a marker and/or selection gene operably linked to a poxviral promoter. Suitable marker or selection genes are, e.g., the genes encoding the green fluorescent protein, β -galactosidase, neomycin-phosphoribosyltransferase or other markers. The use of selection or marker cassettes simplifies the identification and isolation of the generated recombinant poxvirus. However, a recombinant poxvirus can also be identified by PCR technology. Subsequently, a further cell can be infected with the recombinant poxvirus obtained as described above and transfected with a second vector comprising a second foreign or heterologous gene or genes. In case, this gene shall be introduced into a different insertion site of the poxviral genome, the second vector also differs in the poxvirus-homologous sequences directing the integration of the second foreign gene or genes into the genome of the poxvirus. After homologous recombination has occurred, the recombinant virus comprising two or more foreign or heterologous genes can be isolated. For introducing additional foreign genes into the recombinant virus, the steps of infection and transfection can be repeated by using the recombinant virus isolated in previous steps for infection and by using a further vector comprising a further foreign gene or genes for transfection.

[0118] Alternatively, the steps of infection and transfection as described above are interchangeable, i.e., a suitable cell can at first be transfected by the plasmid comprising the foreign gene and, then, infected with the poxvirus. As a further alternative, it is also possible to introduce each foreign gene into different viruses, co-infect a cell with all the obtained

recombinant viruses and screen for a recombinant including all foreign genes. A third alternative is ligation of DNA genome and foreign sequences in vitro and reconstitution of the recombined vaccinia virus DNA genome using a helper virus. A fourth alternative is homologous recombination in *E. coli* or another bacterial species between a poxvirus genome cloned as a bacterial artificial chromosome (BAC) and a linear foreign sequence flanked with DNA sequences homologous to sequences flanking the desired site of integration in the vaccinia virus genome.

[0119] The one or more nucleic acids of the present disclosure may be inserted into any suitable part of the poxvirus. In a preferred aspect, the poxvirus used for the present invention include MVA and/or fowlpox virus. Suitable parts of the MVA and fowlpox virus are non-essential parts of the MVA and the fowlpox genomes.

[0120] For MVA, Non-essential parts of the MVA genome may be intergenic regions or the known deletion sites 1-6 of the MVA genome. Alternatively or additionally, non-essential parts of the recombinant MVA can be a coding region of the MVA genome which is non-essential for viral growth. However, the insertion sites are not restricted to these preferred insertion sites in the MVA genome, since it is within the scope of the present invention that the nucleic acids of the present invention (e.g., MUC1, CEA, and TRICOM) and any accompanying promoters as described herein may be inserted anywhere in the viral genome as long as it is possible to obtain recombinants that can be amplified and propagated in at least one cell culture system, such as Chicken Embryo Fibroblasts (CEF cells).

[0121] Preferably, the nucleic acids of the present invention may be inserted into one or more intergenic regions (IGR) of the MVA and/or fowlpox virus. The term “intergenic region” refers preferably to those parts of the viral genome located between two adjacent open reading frames (ORF) of the MVA and/or fowlpox virus genome, preferably between two essential ORFs of the MVA and/or fowlpox virus genome. For MVA, in certain embodiments, the IGR is selected from IGR 07/08, IGR 44/45, IGR 64/65, IGR 88/89, IGR 136/137, and IGR 148/149. For fowlpox virus, the IGR is selected from BamH1,

[0122] For MVA virus, the nucleotide sequences may, additionally or alternatively, be inserted into one or more of the known deletion sites, i.e., deletion sites I, II, III, IV, V, or VI of the MVA genome. The term “known deletion site” refers to those parts of the MVA genome that were deleted through continuous passaging on CEF cells characterized at 20 passage 516 with respect to the genome of the parental virus from which the MVA is derived from, in particular the parental chorioallantois vaccinia virus Ankara (CVA) e.g., as described in Meisinger-Henschel et al. (2007), *Journal of General Virology* 88:3249-3259.

Vaccines

[0123] In certain embodiments, the recombinant poxviruses of the present disclosure can be formulated as part of a vaccine. For the preparation of vaccines, the poxvirus can be converted into a physiologically acceptable form. In certain embodiments, such preparation is based on experience in the preparation of poxvirus vaccines used for vaccination against smallpox, as described, for example, in Stickl, H. et al., Dtsch. med. Wschr. 99, 2386-2392 (1974).

[0124] An exemplary preparation follows. Purified virus is stored at -80°C with a titer of 5×10^8 TCID₅₀/ml formulated in 10 mM Tris, 140 mM NaCl, pH 7.4. For the preparation of vaccine shots, e.g., 10^2 - 10^8 particles of the virus can be lyophilized in phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be prepared by stepwise, freeze-drying of the virus in a formulation. In certain embodiments, the formulation contains additional additives such as mannitol, dextran, sugar, glycine, lactose, polyvinylpyrrolidone, or other additives, such as, including, but not limited to, antioxidants or inert gas, stabilizers or recombinant proteins (e.g. human serum albumin) suitable for *in vivo* administration. The ampoule is then sealed and can be stored at a suitable temperature, for example, between 4°C and room temperature for several months. However, as long as no need exists, the ampoule is stored preferably at temperatures below -20°C.

[0125] In various embodiments involving vaccination or therapy, the lyophilisate is dissolved in 0.1 to 0.5 ml of an aqueous solution, preferably physiological saline or Tris buffer, and administered either systemically or locally, i.e., by parenteral, subcutaneous, intravenous, intramuscular, intranasal, intradermal, or any other path of administration known to a skilled practitioner. Optimization of the mode of administration, dose, and number of administrations is within the skill and knowledge of one skilled in the art.

[0126] In certain embodiments, attenuated vaccinia virus strains are useful to induce immune responses in immune-compromised animals, e.g., monkeys (CD4<400/ μ l of blood) infected with SIV, or immune-compromised humans. The term "immune-compromised" describes the status of the immune system of an individual that exhibits only incomplete immune responses or has a reduced efficiency in the defense against infectious agents.

Kits, Compositions, and Methods of Use

[0127] In one various embodiments, the invention encompasses kits and/or compositions comprising a recombinant poxvirus that includes the nucleic acids described herein. Preferably, the composition is a pharmaceutical or immunogenic composition.

[0128] In one embodiment, there is a kit and/or composition comprising a combination of two or more recombinant poxviruses each recombinant poxvirus including the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure. The combination comprises a) an orthopoxvirus, such as vaccinia, MVA, MVA-BN, or derivatives of MVA-BN including the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure and b) an avipoxvirus, such as fowlpox, including the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure. It is contemplated that the orthopoxvirus and fowlpox virus combination can be administered as a homologous or heterologous prime-boost regimen.

[0129] In another embodiment, the kit and/or composition including the combination of two or more recombinant poxviruses comprises a) an MVA virus include the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure and b) an avipoxvirus, such as fowlpox, including the MUC1, CEA, and/or TRICOM nucleic acids of the present disclosure. It is contemplated that the MVA virus and fowlpox virus combination can be administered as a homologous or heterologous prime-boost regimen.

[0130] In additional embodiment, each of the one or more recombinant poxviruses further comprise one or more of the costimulatory molecules of the present disclosure. In a preferred embodiment, one or more costimulatory molecules are one or more of the TRICOM molecules of the present disclosure.

[0131] It is contemplated that the kit and/or composition can comprise one or multiple containers or vials of the recombinant poxviruses of the present disclosure, together with instructions for the administration of the recombinant poxviruses.

[0132] The kits and/or compositions provided herein may generally include one or more pharmaceutically acceptable and/or approved carriers, additives, antibiotics, preservatives, adjuvants, diluents and/or stabilizers. Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like.

[0133] For the preparation of compositions (e.g., pharmaceutical and/or immunogenic compositions), the recombinant poxviruses provided herein can be converted into a physiologically acceptable form. This can be done based on experience in the preparation of

poxvirus vaccines used for vaccination against smallpox as described by H. Stickl et al., Dtsch. med. Wschr. 99:2386-2392 (1974).

[0134] For example, purified viruses can be stored at -80°C with a titer of 5×10^8 TCID₅₀/ml formulated in about 10 mM Tris, 140 mM NaCl pH 7.4. For the preparation of vaccine shots, e.g., 10^2 - 10^8 or 10^2 - 10^9 particles of the virus can be lyophilized in 100 ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other aids such as antioxidants or inert gas, stabilizers or recombinant proteins (e.g., human serum albumin) suitable for in vivo administration. A typical virus containing formulation suitable for freeze-drying comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l Dextran (MW 36,000-40,000), 45 g/l Sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. The glass ampoule is then sealed and can be stored between 4°C and room temperature for several months. However, as long as no need exists, the ampoule is stored preferably at temperatures at or below -20°C .

[0135] For vaccination or therapy, the lyophilisate can be dissolved in an aqueous solution (e.g., 0.1 to 0.5 ml), preferably water for injection, physiological saline or Tris buffer, and administered either systemically or locally, i.e., parenteral, subcutaneous, intravenous, intramuscular, intranasal, or any other path of administration known to the skilled practitioner. The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner.

[0136] In various other embodiments, there are one or more methods related to generating and/or improving the stability of the recombinant poxvirus and/or the transgenes therein throughout successive passaging of the recombinant poxvirus. In a more specific embodiment, the recombinant poxvirus is stable through at least 3 or 4 passages.

[0137] Having a stable recombinant poxvirus throughout multiple passages is particularly important for many reasons, some of which include large scale production of the recombinant virus and its use as a medicament, as well as governmental policies for vaccine stability through multiple passages. For recombinant poxviruses of the present invention, generating a stable recombinant poxvirus through at least 3 or 4 passages is important as PANVAC-V and PANVAC-F began to demonstrate instability and/or loss of transgene viability around passage 1 (*See, e.g.*, Tables 1 and 2; and Figures 1A and 1B).

[0138] In one embodiment there is a method for generating a poxvirus having a MUC1 transgene that is stable through successive passaging of the recombinant poxvirus, the method comprising: a) providing any one of the nucleic acids or expression cassettes of the present disclosure; and b) inserting the nucleic acid or the expression cassette into a recombinant poxvirus, wherein the recombinant poxvirus is stable through successive passaging.

Exemplary Methods according to the present disclosure

1. In another embodiment, there is a method for generating a recombinant poxvirus that is stable through successive passaging of the recombinant poxvirus, the method comprising: a) providing a first nucleic acid encoding a MUC1 peptide having at least two Variable N-Terminal Repeat (VNTR) domains, wherein a) the arrangement of the at least two VNTR domains are shuffled, and b) the at least two VNTR domains are codon optimized, wherein the recombinant poxvirus is stable through successive passaging of the recombinant poxvirus.

2. In another embodiment, there is a method for generating a stable recombinant poxvirus that is stable through successive passaging of the recombinant poxvirus, the method comprising: providing a first nucleic acid encoding a MUC1 protein, the MUC1 protein comprising at least two VNTR domains; shuffling or rearranging the order of the at least two VNTR domain repeats; optimizing the codons of the at least two VNTR domain repeats; inserting the first nucleic acid sequence into the poxvirus to generate a recombinant poxvirus that is stable successive passaging of the recombinant poxvirus.

3. The methods of any one of 1 and 2 wherein the first nucleic acid is at least 95% homologous to SEQ ID NO:2, 95% homologous to SEQ ID NO: 4, 95% homologous to SEQ ID NO: 3, or 95% homologous to SEQ ID NO: 5.

4. The method of any one of 1 to 3, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 2.

5. The method of any one of 1 to 4, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 3.

6. The method of any one of 1 to 5, wherein the nucleic acid comprises SEQ ID NO: 2.

7. The method of any one of 1 to 6, wherein the nucleic acid comprises SEQ ID NO: 5.

8. The method of any one of 1 to 7, wherein the method further comprises substituting at least one nucleotide in a repetitive nucleotide region of a second nucleic acid encoding a CEA peptide, wherein the repetitive nucleotide region is defined as: (i) three or more

consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides; and inserting the second nucleic acid in the recombinant poxvirus.

9. The method of 8, wherein the repetitive region of the second nucleic acid is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

10. The method of any one of 8 and 9, wherein the repetitive nucleotide region of the second nucleic acid is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

11. In one aspect of the methods of 1-10, the CEA nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

12. In one aspect of the methods of 1-11, the CEA repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

13. In one aspect of the methods of 1-12, the substitution is to at least 2, 3, 4, 5, or 10 repetitive nucleotide regions of the CEA nucleic acid.

14. In one aspect of the methods of 1-13, the CEA nucleic acid comprises SEQ ID NO: 14.

15. In one aspect of the methods of 1-14, the method further comprises substituting at least one nucleotide in a repetitive nucleotide region of a nucleic acid encoding a costimulatory molecule selected from B7-1, ICAM-1, and/or LFA-3, CEA, wherein the repetitive nucleotide region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides; and inserting the nucleic acid encoding a costimulatory molecule in the recombinant poxvirus.

16. In one aspect of the methods of 1-15, the costimulatory molecule repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

17. In one aspect of the methods of 1-16, the costimulatory molecule repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

18. In one aspect of the methods of 1-17, the costimulatory molecule repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

19. In one aspect of the methods of 1-18, the substitution is to at least 2, 3, 4, 5, or 10 repetitive nucleotide regions of the costimulatory molecule nucleic acid.

20. In one aspect of the methods of 1-19, the nucleic acid encoding the costimulatory molecule is selected from B7-1 (SEQ ID NOs: 15-17); ICAM-1 (SEQ ID NOs: 18-20) and LFA-3 (SEQ ID NOs: 21-23).

21. In one aspect of the methods of 1-20, the nucleic acid encoding the costimulatory molecule is at least 80%, 85%, 90%, or 95% homologous to at least one of B7-1 (SEQ ID NO: 15; ICAM-1 (SEQ ID NO: 18) and LFA-3 (SEQ ID NO: 21).

22. In one aspect of the methods of 1-21, the nucleic acid encoding the costimulatory molecule is at least 80%, 85%, 90%, or 95% homologous to at least one of B7-1 (SEQ ID NO: 17; ICAM-1 (SEQ ID NO: 20) and LFA-3 (SEQ ID NO: 23).

23. In one aspect of the methods of 1-22, the nucleic acid encoding the costimulatory molecule comprises at least one of B7-1 (SEQ ID NO: 17; ICAM-1 (SEQ ID NO: 20) and LFA-3 (SEQ ID NO: 23).

24. In one aspect of the methods of 1-23, the nucleic acid encoding the costimulatory molecule is comprises: B7-1 (SEQ ID NO: 15; ICAM-1 (SEQ ID NO: 18) and LFA-3 (SEQ ID NO: 21).

25. In one aspect of the methods of 1-24, the first nucleic acid encoding the MUC1 is selected from SEQ ID NOs: 31, 32, 33, and 34.

26. As provided for by the present disclosure, the recombinant poxvirus of the methods of 1-26, can be selected from an orthopoxvirus or an avipoxvirus. In preferred embodiments, the orthopoxvirus is selected from a vaccinia virus, MVA, MVA-BN, and derivatives of MVA-BN. In a more preferred embodiment, the orthopoxvirus is either MVA, MVA-BN, or a derivative of MVA-BN. In still another more preferred embodiment, the avipoxvirus is a fowlpox virus.

[0139] In other embodiments, there is a use of a) a nucleic acid, b) an expression cassettes, c) a composition, d) a host cell, or e) a vector according to the present disclosure in a method for generating a recombinant poxvirus that is stable through successive passaging of the poxvirus.

[0140] In still other embodiments, there is a use of a) a recombinant poxvirus, b) a nucleic acid, c) an expression cassette, d) a composition, d) a host cell, or e) a vector according to the present disclosure in the preparation of a medicament preferably a vaccine.

[0141] In still further embodiments, there is a recombinant poxvirus, b) a nucleic acid, b) an expression cassette, c) a composition, d) a host cell, or e) a vector according to the present disclosure for use as a medicament preferably a vaccine.

[0142] In yet additional embodiments, there is a recombinant poxvirus, b) a nucleic acid, b) an expression cassette, c) a composition, d) a host cell, or e) a vector according to the present disclosure for use in a method for introducing a coding sequence into a target cell.

EXAMPLES

[0143] The following examples illustrate the invention but, of course should not be construed as in any way limiting the scope of the claims.

Example 1: Construction of Recombinant Poxviruses

[0144] Generation of the poxviruses encoding MUC1(e.g., mBN399, mBN400, mBN336, mBN373, and mBN420) was done by insertion of the indicated MUC1 and CEA nucleic acid sequences with their promoters via simultaneous infection and transfection of CEF cultures, followed by allowed homologous recombination between the viral genome and the recombination plasmid pBN146. Insert-carrying virus was isolated, characterized, and virus stocks were prepared.

[0145] For construction of mBN398 and mBN400, an MVA recombination plasmid containing homologous sequences which are also present in Vaccinia Virus at the IGR88/89 were used). The MUC1 and CEA nucleotide sequence was inserted between the Vaccinia Virus sequences at IGR 88/89 to allow for recombination into the Vaccinia viral genome. Thus, a plasmid was constructed that contained the MUC1 and CEA nucleotide sequence downstream of a poxvirus promoter. For mBN 398 and mBN400 SEQ ID NO: 1 (MUC1) and SEQ ID NO: 13 (CEA) were used. Promoters for MUC1 and CEA in mBN398 were PrS promoter (MUC1) and the 40k-MVA1 promoter (CEA), respectively. Promoters for MUC1 and CEA in mBN400 were Pr13.5long (MUC1) and the PrS5E promoter (CEA), respectively. Costimulatory molecules of TRICOM were included as part of mBN398 and mBN400. These sequences included: B7-1, ICAM-1, and LFA-3 and comprise SEQ ID NOs: 16, 19, and 21, respectively.

[0146] For construction of mBN336, three recombination plasmids were used for the three transgenes pBN 374 (forTRICOM), pBN 515 (for CEA SEQ ID NO: 13) , pBN 525 (for MUC1 SEQ ID NO: 2), insert sequences which are also present in MVA (IGR88/89(MUC1), IGR 44/45 (CEA), IGR 148/149 (TRICOM). The MUC1 and CEA nucleotide sequence was inserted between the MVA virus sequences to allow for recombination into the MVA viral genome. Thus, a plasmid was constructed that contained the MUC1 and CEA nucleotide sequence downstream of a poxvirus promoter. For mBN336, SEQ ID NO: 2 (MUC1) and SEQ ID NO: 13 (CEA) were used. Promoters were PrS promoter (for MUC1) and the 40k promoter (for CEA). Costimulatory molecules of TRICOM were included as part of mBN336. These sequences included: B7-1, ICAM-1, and LFA-3 and comprise SEQ ID NOs: 17, 20, and 23, respectively. pBN632 contains sequences which are also present in MVA (within IGR 88/89). The MUC1 and CEA nucleotide sequence was inserted between the MVA virus sequences to allow for recombination into the MVA viral genome. Thus, a plasmid was constructed that contained the MUC1 and CEA nucleotide sequence downstream of a poxvirus promoter. For mBN420, SEQ ID NO: 5 (MUC1) and SEQ ID NO: 14 (CEA) were used. Promoters for MUC1 and CEA were Pr13.5 promoter (see US patent publication 2015/0299267) (MUC1) and the 40k MVA1 promoter (CEA), respectively. Costimulatory molecules of TRICOM were included as part of mBN420 and integrated within IGR 88/89. These sequences included: B7-1, ICAM-1, and LFA-3 and comprise SEQ ID NOs: 15, 18, and 21, respectively.

[0147] For construction of mBN373, recombination plasmid pBN563 contains sequences which are also present in fowlpox virus. The MUC1 and CEA nucleotide sequence was inserted between the fowlpox virus sequences in the BamH1 region to allow for recombination into the fowlpox viral genome. Thus, a plasmid was constructed that contained the MUC1 and CEA nucleotide sequence downstream of a poxvirus promoter. For mBN373, SEQ ID NO: 3 (MUC1) and SEQ ID NO: 14 (CEA) were used. Promoters for MUC1 and CEA were 40K FPV-1 PrS promoter (MUC1) and the 40k-MVA1 promoter (CEA), respectively. Costimulatory molecules of TRICOM were included as part of mBN373. These sequences included: B7-1, ICAM-1, and LFA-3 and comprise SEQ ID NOs: 15, 18, and 21, respectively.

[0148] The above recombination plasmids also contained a selection cassette comprising a synthetic vaccinia virus promoter (Ps), a drug resistance gene GPT, an internal ribosomal entry site (IRES), and the enhanced green fluorescent protein (EGFP), and the drug

resistance gene guanine-xanthine phosphoribosyltransferase (Ecogpt) in combination with the Monomeric Red Fluorescent Protein. All selection genes (GFP, NPTII, and mRFP1) were encoded by a single bicistronic transcript.

[0149] CEF cultures were inoculated with Vaccinia virus for mBN399/400, MVA-BN for mBN336, mBN420, or FPV for mBN373 and each CEF culture was also transfected with plasmid DNA. In turn, samples from these cell cultures were inoculated into CEF cultures in medium containing selection drugs, and EGFP-expressing viral clones were isolated by plaque purification. Virus stocks which grew in the presence of the selection drugs and expressed EGFP were designated one of the following: mBN399, mBN400 (Vaccinia viruses), mBN336, mBN420 (MVA virus), and mBN373 (fowlpox). Generation of the recombinant viruses and preparation of the virus stock involved between 5-12 sequential passages, including one (1) to five (5) plaque purifications.

[0150] The recombinant poxviruses were passaged in CEF cell cultures in the absence of selection drugs. The absence of selection drugs allowed loss of the region encoding the selection genes, gpt and EGFP and the associated promoter (the selection cassette) from the inserted sequence. Recombination resulting in loss of the selection cassette is mediated by the F1 I4L region and a subsection of that region, the F1 repeat (F1 rpt), which flank the selection cassette in plasmid of each construct. These duplicated sequences were included to mediate recombination that results in loss of the selection cassette, leaving only the MUC1 and CEA sequences inserted in the described intergenic regions of the constructs described herein

[0151] Plaque-purified virus lacking the selection cassette was prepared. Such preparation involved fifteen (15) passages including five (5) plaque purifications.

[0152] The presence of the MUC1 and CEA sequence and absence of parental MVA-BN virus in mBN336, mBN420, and mBN373 stocks was confirmed by PCR analysis, and nested PCR was used to verify the absence of the selection cassette (the gpt and EGFP genes/ NPTII and mRFP1).

[0153] Expression of the MUC1 and CEA proteins was demonstrated in cells inoculated with MVA-BN-MUC1-CEA-TRICOM *in vitro*.

Example 2: PCR analysis of MVA-mBN336 passages 1-7

[0154] Genetic stability of MVA-mBN336B was evaluated by cultivation for seven passages. MVA-mBN336B encodes 5 human transgenes, with human Mucin 1 (MUC-1)

and human Carcinoembryonic Antigen (CEA) being the target antigens of this vaccine candidate, and 3 genes encoding human immune costimulatory molecules (designated TRIad of COstimulatory Molecules, or TRICOM) as support for induction of a robust and directed immune response: leukocyte function-associated antigen-3 (LFA-3), intracellular adhesion molecule 1 (ICAM-1), and B7-1. The transgenes were inserted into three intergenic regions (IGR) of MVA-BN®: IGR 44/45 containing CEA, IGR 88/89 containing hMUC1, and IGR 148/149 containing the TRICOM genes. Transgene expression is driven by the pox virus promoters 40k-MVA1, 30k, I3L and PrS.

[0155] Primary chicken embryo fibroblast (CEF) cells were prepared, seeded in roller bottles (RB) (7×10^7 cells) in VP-SFM medium and incubated for 4 days at 37° C. VP-SFM medium was replaced by 100 ml RPMI medium and the cells were infected with a MOI of approximately .3-00.1 referring to a cell number of 1×10^8 cells/RB and cultivated for 3 days at 30°C. After incubation, virus samples were harvested by freezing the RB at -20°C for at least 16 h, followed by thawing of the RB to collect the cell virus suspension. The exact volume of the cell suspension was determined, virus samples were sonicated and subsequently aliquoted and stored at -80°C. This procedure was repeated six times resulting in seven passages.

[0156] PCR analysis of the inserted transgenes was performed for each passage after cultivation at 30°C. Figure 9A shows the PCR results for stability of CEA over seven passages. Figure 9B shows the PCR results for stability of MUC1 over seven passages. Figure 9C shows the PCR results for the stability of the TRICOM over 7 passages. The recombination plasmids used for generation of MVA-mBN336B were used as positive controls, MVA-BN® was used as negative control (empty vector backbone) and H₂O was used as control for the PCR reaction.

[0157] Figure 10A and 10B illustrates an analysis of Passage 7 sample. Figure 10A is a PCR amplification of Passage 7 samples sent for analysis by sequencing. Individual PCR amplifications were performed for each individual transgene: CEA, MUC1, and TRICOM. B) Electropherograms of the MUC1 nt-sequence depicting the loci containing the detected point mutation leading to a frame shift. The point mutation was detected in passage 5 for the first time PCR amplification and an Electropherogram of the MUC1 nt-sequence depicting the loci containing the detected point mutation leading to a frame shift. The point mutation was detected in passage 5 for the first time is an Electropherogram analyzing mutations occurring in passages 5, 6, and 7.

[0158] Shown in Figures 9 and 10, the MUC1, CEA, and TRICOM combination in mBN336 demonstrated an improved and increased stability as compared to MUC1, CEA, and TRICOM transgenes in PANVAC-V and PANVAC-F (*compare, e.g.,* Figure 1 and Tables 1, and 2 with Figures 3 and 4). Starting at Passage 5, a frameshift mutation was detected within a minor population of the analyzed material.

[0159] The stability illustrated through passage 4 demonstrates the ability of the MVA-mBN336 to overcome the stability problems associated with PANVAC and other attempts to generate a stable poxvirus including MUC1. The stability of MVA-mBN336 is additionally advantageous, as manufacture and larger scale production of MVA-based vaccines are typically taken from MVAs at passage 3 or passage 4. Thus, because MVA-mBN336 is stable through passage 4, large scale production can begin and significant regulatory hurdles with regard to stability can be overcome.

Example 3: Improved Stability of FPV-mBN373

[0160] Genetic stability of FPV-mBN373B was evaluated over seven passages. Cultivation was performed in roller bottles (RB) as applied during large scale production used for manufacture of clinical trial material. Each passage was analysed for virus titer by flow cytometry assay and the correct size of the transgene insert by PCR. In addition, the last passage (P7) was analysed by sequencing of the transgenes.

[0161] Primary chicken embryo fibroblast (CEF) cells were prepared, seeded in RBs (7×10^7 cells/RB) in VP-SFM medium and incubated for 3 days at 37°C. The VP-SFM medium was replaced by 100 ml RPMI medium and the cells were infected with a MOI of 0.1 referring to a cell number of 1×10^8 /RB and cultivated for 4 days at 37°C. After incubation, virus samples were harvested by freezing the RB at -20°C for at least 16 h, followed by thawing of the RB to collect the cell virus suspension. The exact volume of the cell suspension was determined, virus samples were sonicated, and subsequently aliquoted and stored at 80°C. The infectious virus titer was determined after each passage to monitor the virus titers and to enable the infection of the next passage with a defined MOI. This procedure was repeated six times resulting in seven passages.

[0162] Shown in Figure 11A, PCR analysis of the inserted transgenes was performed for each passage after cultivation at 37°C. The recombination plasmid used for generation of FPV-mBN373B was used as positive control, FPV was used as negative control (empty vector backbone) and H₂O was used as control for the PCR reaction.

[0163] Shown in Figure 11B, sequencing of the seventh passage was performed after amplification of the BamHI J site containing the transgenes and at least 600 bp of each flanking region. The PCR amplicon of FPV-mBN373B analysed at passage seven (37°C) resulted in the expected band size of 5566 bp (PCR1) and 5264 bp (PCR2), covering the inserted transgenes and at least 600 bp of each flanking region. The results showed a 100% identity of the assembled sequence compared to the theoretical sequence, confirming the genetic stability of FPV-mBN373B for 7 passages at 37°C.

[0164] In at least one aspect, the resulting stability of the MUC1 transgene, SEQ ID NO: 3, in mBN373 was surprising as both mBN373 and mBN336, include SEQ ID NO:3. Accordingly, while MUC1 of SEQ ID NO: 3 begins to show instability at Passage 5 in mBN336 (MVA virus), the same SEQ ID NO:3 is stable in mBN373 (fowlpox virus) at least until passage 7.

Example 4: Stability of MVA-mBN420

[0165] Genetic stability of MVA-mBN 420 was evaluated over seven passages. Cultivation was performed in roller bottles (RB) as applied during large scale production used for manufacture of clinical trial material. The study was performed at 30°C and 34°C using an MOI of approximately 0.05 to 0.1 and a virus incubation period of 4 days as these conditions are representative for a typical large scale production used for manufacture of clinical trial material. Each passage was analysed for virus titer by flow cytometry assay and the correct size of the transgene insert by PCR.

[0166] Primary chicken embryo fibroblast (CEF) cells were prepared, seeded in RBs (7×10^7 cells/RB) in VP-SFM medium and incubated for 3 days at 37°C. The VP-SFM medium was replaced by 100 ml RPMI medium and the cells were infected with a MOI of 0.05 to 0.1 referring to a cell number of 1×10^8 /RB and cultivated for 4 days at 30°C and 34°C. After incubation, virus samples were harvested by freezing the RB at -20°C for at least 16 h, followed by thawing of the RB to collect the cell virus suspension. The virus samples were sonicated, and subsequently aliquoted and stored at 80°C. The infectious virus titer was determined after each passage to monitor the virus titers and to enable the infection of the next passage with a defined MOI. This procedure was repeated six times resulting in seven passages.

[0167] PCR analysis of the inserted transgenes was performed for each passage after cultivation at 30°C 4. The results of passaging performed at 30°C are shown in Figure 12. The recombination plasmid used for generation of mBN420 was used as positive control,

MVA-BN was used as negative control (empty vector backbone) and H₂O was used as control for the PCR reaction.

[0168] Shown in Figure 12, the stability of the MVA in mBN420 was decreased as compared to the MVA in mBN336 and the fowlpox virus in mBN373.

Example 5: Improved Stability of additional recombinant MVA and recombinant Fowlpox viruses encoding MUC1 and CEA

[0169] Generation of additional recombinant MVAs and recombinant fowlpox viruses of the present invention is conducted as described in Example 1. Nucleic acids encoding MUC1, CEA, and TRICOM transgenes comprising SEQ ID NOs: 31, 32, 33, or 34 (for MUC1) and SEQ ID NOs: 13 or 14 (for CEA) are inserted into MVA-BN as described in Example 1. Additionally, TRICOM is inserted into the MVA, the TRICOM sequences including SEQ ID NOs: 15 or 17 (for B7.1), SEQ ID NOs: 18 or 20 (for ICAM-1), and SEQ ID NOs: 21 or 23 (for LFA-3) are inserted into the MVA as described in Example 1.

[0170] Additionally, nucleic acids encoding MUC1 and CEA transgenes comprising SEQ ID NOs: 31, 32, 33, or 34 (for MUC1) and SEQ ID NOs: 13 or 14 (for CEA) are inserted into MVA-BN as described in Example 1. Additionally, TRICOM is inserted into the fowlpoxvirus, the TRICOM sequences including SEQ ID NOs: 15 or 17 (for B7.1), SEQ ID NOs: 18 or 20 (for ICAM-1), and SEQ ID NOs: 21 or 23 (for LFA-3) are inserted into the fowlpox as described in Example 1.

[0171] SEQ ID NOs: 31, 32, 33, or 34 each encode a MUC1 peptide comprising SEQ ID NO: 35.

[0172] The novel MUC1 nucleic acids of SEQ ID NOs: 31, 32, 33, and 34 each encode variations of the nucleic acids of the present invention without the agonist epitopes from WO 2013/103658. In several aspects, substitution and/or removal of the agonist epitopes do not affect stability of the recombinant poxviruses of the present invention, as the presence of the agonist epitopes function to enhance immunogenicity of the MUC1 rather than stability or instability.

[0173] Expression of the MUC1, CEA, and TRICOM proteins is demonstrated in cells inoculated with MVA-BN-MUC1-CEA-TRICOM *in vitro* as described in Example 1.

[0174] Improved genetic stability of transgenes in MVA and/or Fowlpox viruses is evaluated over seven passages. Cultivation is performed in roller bottles (RB) as applied during large scale production used for manufacture of clinical trial material. The study is performed at 30°C, 34°C or 37°C (depending on the vector system used) using an MOI of

approximately 00.05-00.1 and a virus incubation period of 2, 3, 4, 5, 6, or 7 days as these conditions are representative for a typical large scale production used for manufacture of clinical trial material. Each passage is analysed for virus titer by flow cytometry assay and the correct size of the transgene insert by PCR. In addition, the last passage (P7) is analysed by sequencing of the transgenes.

[0175] Primary chicken embryo fibroblast (CEF) cells are prepared, seeded in RBs (7×10^7 cells/RB) in VP-SFM medium and incubated for 3 days at 37°C. The VP-SFM medium is replaced by 100 ml RPMI medium and the cells are infected with a MOI of 0.005 to 0.1 and cultivated for 4 days at 30°C, 34°C or 37°C (depending on the vector system used). After incubation, virus samples are harvested by freezing the RB at -20°C for at least 16 h, followed by thawing of the RB to collect the cell virus suspension. The virus samples are sonicated, and subsequently aliquoted and stored at 80°C. The infectious virus titer is determined after each passage to monitor the virus titers and to enable the infection of the next passage with a defined MOI. This procedure is repeated six times resulting in seven passages.

[0176] PCR analysis of the inserted transgenes is performed for each passage after cultivation at 30°C, 34°C or 37°C (depending on the vector system). The recombination plasmid used for generation of each corresponding poxvirus (e.g., MVA-BN or fowlpox virus) is used as positive control, MVA-BN or fowlpoxvirus is used as negative control (empty vector backbone) and H₂O is used as control for the PCR reaction.

[0177] Sequencing of the seventh passage is performed after amplification of the IGR site containing the transgenes and at least 600 bp of each flanking region. The PCR amplicon of each construct is analysed at passage seven. Sequencing results of the MUC1, CEA and/or TRICOM nucleic acids are conducted to verify that the MVA and/or fowlpox virus is stable among the transgenes.

[0178] It will be apparent that the precise details of the methods or compositions described herein may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

CLAIMS

We claim:

1. A recombinant poxvirus which is stable through successive passaging of the recombinant poxvirus, the recombinant poxvirus comprising a first nucleic acid encoding a MUC1 peptide having at least two Variable N-Terminal Repeat (VNTR) domains, wherein a) the arrangement of the at least two VNTR domains are shuffled, and b) the at least two VNTR domains are codon optimized, wherein the recombinant poxvirus is stable through successive passaging of the recombinant poxvirus.
2. The recombinant poxvirus of claim 1, wherein the first nucleic acid comprises at least three VNTR domains.
3. The recombinant poxvirus of any one of claims 1-2, wherein the order of the at least two VNTR domains are shuffled as compared to SEQ ID NO: 6.
4. The recombinant poxvirus of any one of claims 1-3, wherein the presence of a) and b) improves stability through successive passaging as compared to PANVAC.
5. The recombinant poxvirus of any one of claims 1-4 wherein the first nucleic acid sequence comprises a nucleic acid sequence having at least 95%, 96%, 97%, or 98% identity to SEQ ID NO:2.
6. The recombinant poxvirus of any one of claims 1-5, wherein the first nucleic acid sequence comprises SEQ ID NO:2.
7. The recombinant poxvirus of any one of claims 1-6, further comprising a second nucleic acid encoding a carcinoembryonic antigen (CEA).
8. The recombinant poxvirus of claim 7, wherein the second nucleic acid comprises SEQ ID NO:13.
9. The recombinant poxvirus of claim 7, wherein the second nucleic acid comprises at least one nucleotide substitution in at least one repetitive nucleotide region of the second nucleic acid, wherein the at least one repetitive nucleotide region is defined as a) three more consecutively repeated G or C nucleotides and/or b) three or more consecutively repeated T nucleotides.
10. The recombinant poxvirus of claim 9, wherein the at least one repetitive nucleotide region is further defined as a) three or more consecutively repeated G nucleotides and/or b) three or more consecutively repeated C nucleotides.

11. The recombinant poxvirus of any one of claims 9-10, wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T nucleotides.

12. The recombinant poxvirus of any one of claims 9-10, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

13. The recombinant poxvirus of any one of claims 9-12, wherein the second nucleic acid comprises at least one substitution to at least 2, 3, 4, 5, or 10 of the repetitive nucleotide regions of the second nucleic acid.

14. The recombinant poxvirus of any one of claims 9-13, wherein the second nucleic acid comprises at least one nucleotide substitution in at least 10, at least 12, at least 15, and/or at least 19 repetitive nucleotide regions of the second nucleic acid

15. The recombinant poxvirus of any one of claims 9-14, wherein the second nucleic acid comprises at least one nucleotide substitution in 19 regions of the second nucleic acid.

16. The recombinant poxvirus of any one of claims 9-14, wherein the second nucleic acid comprises SEQ ID NO: 14.

17. The recombinant poxvirus of any one of claims 1-16, wherein the recombinant poxvirus is selected from an orthopoxvirus and an avipoxvirus.

18. The recombinant poxvirus of claim 17, wherein the orthopoxvirus is a vaccinia virus.

19. The recombinant poxvirus of claim 18, wherein the vaccinia virus is a modified vaccinia virus Ankara (MVA).

20. The recombinant poxvirus of claim 19, wherein the MVA is MVA-BN or a derivative of MVA-BN.

21. The recombinant poxvirus of any one of claims 1-20, wherein the recombinant poxvirus is stable through at least Passage 3 and/or Passage 4.

22. The recombinant poxvirus of claim 17, wherein the avipoxvirus is a fowlpox virus.

23. The recombinant poxvirus of claim 22, wherein the recombinant poxvirus is stable through at least Passage 4, preferably through at least Passage 7.

24. The recombinant poxvirus of any one of claims 1-23, wherein the first nucleic acid further comprises a nucleotide sequence encoding a peptide fragment selected from the group consisting of: YLAPPAHGV (SEQ ID NO:25), YLDTRPAPV (SEQ ID NO:26), YLAIVYLIAl (SEQ ID NO:27), YLIALAVCQV (SEQ ID NO:28), YLSYTNPAV (SEQ ID NO:29), SLFRSPYEK (SEQ ID NO:30), and combinations thereof.

25. The recombinant poxvirus of any one of claims 1-24, wherein the poxvirus further comprises a nucleic acid encoding one or more co-stimulatory molecules.
26. The recombinant poxvirus of claim 25, wherein the one or more co-stimulatory molecules are selected from B7-1, ICAM-1, LFA-3, and combinations thereof.
27. The recombinant poxvirus of claim 26, wherein the B7-1 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 15, 16, or 17.
28. The recombinant poxvirus of any one of claims 26-27, wherein the B7-1 nucleic acid comprises SEQ ID NO: 15, 16, or 17.
29. The recombinant poxvirus of any one of claims 26-28, wherein the B7-1 nucleic acid comprises SEQ ID NO: 17.
30. The recombinant poxvirus of any one of claims 26-28, wherein the B7-1 nucleic acid comprises SEQ ID NO: 15.
31. The recombinant poxvirus of claim 26, wherein the ICAM-1 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 18, 19, or 20.
32. The recombinant poxvirus of any one of claims 26 and 31, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 18, 19, or 20.
33. The recombinant poxvirus of any one of claims 26 and 31-32, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 18.
34. The recombinant poxvirus of any one of claims 26 and 31-32, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 20.
35. The recombinant poxvirus of claim 26, wherein the LFA-3 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 21, 22, or 23.
36. The recombinant poxvirus of claim 35, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 21, 22, or 23.
37. The recombinant poxvirus of any one of claims 35-36, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 21.
38. The recombinant poxvirus of any one of claims 35-36, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 23.
39. A method for generating a recombinant poxvirus that is stable through successive passaging of the recombinant poxvirus, the method comprising:
- providing a first nucleic acid encoding a MUC1 protein having at least two Variable N-Terminal Repeat (VNTR) domains, wherein a) the arrangement of the at least two VNTR domains are shuffled, and b) the at least two VNTR domains are codon optimized, wherein the recombinant poxvirus is stable through successive passaging.

40. The method of claim 39, wherein the first nucleic acid comprises at least three VNTR domains.

41. The method of any one of claims 39-40, wherein the order of the at least two VNTR domains are shuffled as compared to SEQ ID NO: 6.

42. The method of any one of claims 39-41, wherein the presence of a) and b) improves stability through successive passaging as compared to PANVAC.

43. The method of any one of claims 39-42, wherein the first nucleic acid sequence comprises a nucleic acid sequence having at least 95%, 96%, 97%, or 98% identity to SEQ ID NO:2 or SEQ ID NO:3.

44. The method of any one of claims 39-43, wherein the first nucleic acid sequence comprises SEQ ID NO:2 or SEQ ID NO:3.

45. The method of any one of claims 39-44 further comprising a second nucleic acid encoding for a carcinoembryonic antigen (CEA).

46. The method of claim 45, wherein the second nucleic acid comprises SEQ ID NO:13.

47. The method of any one of claims 45-46, wherein the second nucleic acid comprises at least one nucleotide substitution in at least one repetitive nucleotide region of the second nucleic acid, wherein the at least one repetitive nucleotide region is defined as a) three more consecutively repeated G or C nucleotides and/or b) three or more consecutively repeated T nucleotides.

48. The method of any one of claims 45-47, wherein the at least one repetitive nucleotide region is further defined as a) three or more consecutively repeated G nucleotides and/or b) three or more consecutively repeated C nucleotides.

49. The method of any one of claims 45-48, wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T nucleotides.

50. The method of any one of claims 47-49, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

51. The method of any one of claims 47-50, wherein the second nucleic acid comprises at least one substitution to at least 2, 3, 4, 5, or 10 of the repetitive nucleotide regions of the second nucleic acid.

52. The method of any one of claims 47-51, wherein the second nucleic acid comprises at least one nucleotide substitution in at least 10, at least 12, at least 15, and/or at least 19 repetitive nucleotide regions of the second nucleic acid

53. The method of any one of claims 47-52, wherein the second nucleic acid comprises at least one nucleotide substitution in 19 regions of the second nucleic acid.

54. The method of any one of claims 47-53, wherein the second nucleic acid comprises SEQ ID NO: 14.

55. The method of any one of claims 39-54, wherein the recombinant poxvirus is selected from an orthopoxvirus and an avipoxvirus.

56. The method of claim 55, wherein the orthopoxvirus is a vaccinia virus.

57. The method of claim 56, wherein the vaccinia virus is a modified vaccinia virus Ankara (MVA).

58. The method of claim 57, wherein the MVA is MVA-BN, or a derivative of MVA-BN.

59. The method of any one of claims 39-58, wherein the recombinant poxvirus is stable through at least Passage 3 or Passage 4.

60. The method of claim 55, wherein the avipoxvirus is a fowlpox virus.

61. The method of claim 60, wherein the recombinant poxvirus is stable through at least Passage 3 or Passage 4.

62. The method of any one of claims 39-61, wherein the first nucleic acid further comprises a nucleotide sequence encoding a peptide fragment selected from the group consisting of: YLAPPAHGV (SEQ ID NO:25), YLDTRPAPV (SEQ ID NO:26), YLAIVYLIAl (SEQ ID NO:27), YLIALAVCQV (SEQ ID NO:28), YLSYTNPAV (SEQ ID NO:29), SLFRSPYEK (SEQ ID NO:30), and combinations thereof.

63. The method of any one of claims 39-62, wherein the poxvirus further comprises a nucleic acid encoding one or more co-stimulatory molecules.

64. The method of claim 63, wherein the one or more co-stimulatory molecules are selected from B7-1, ICAM-1, LFA-3, and combinations thereof.

65. The method of claim 64, wherein the B7-1 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 15, 16, or 17.

66. The method of any one of claims 64-65, wherein the B7-1 nucleic acid comprises SEQ ID NO: 15, 16, or 17.

67. The method of any one of claims 64-66, wherein the B7-1 nucleic acid comprises SEQ ID NO: 17.

68. The method of any one of claims 64-66, wherein the B7-1 nucleic acid comprises SEQ ID NO: 15.

69. The method of claim 64, wherein the ICAM-1 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 18, 19, or 20.

70. The method of any one of claims 64 and 69, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 18, 19, or 20.

71. The method of any one of claims 64 and 69-70, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 18.

72. The method of any one of claims 64 and 69-70, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 20.

73. The method of claim 64, wherein the LFA-3 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 21, 22, or 23.

74. The method of any one of claims 64 and 73, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 21, 22, or 23.

75. The method of any one of claims 64 and 73-74, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 21.

76. The method of any one of claims 64 and 73-74, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 23.

77. A nucleic acid encoding a MUC1 peptide having at least two Variable N-Terminal Repeat (VNTR) domains, wherein a) the arrangement of the at least two VNTR domains are shuffled, and b) the at least two VNTR domains are codon optimized, wherein the presence of the nucleic acid as part of a recombinant poxvirus improves the stability of the nucleic acid and/or the recombinant poxvirus through successive passaging as compared to a recombinant poxvirus encoding a MUC1 peptide without a) and b).

78. The nucleic acid of claim 77, wherein the first nucleic acid comprises at least three VNTR domains.

79. The nucleic acid of any one of claims 77-78, wherein the order of the at least two VNTR domains are shuffled as compared to SEQ ID NO: 6.

80. A nucleic acid encoding a MUC1 peptide, the nucleic acid comprising a nucleotide substitution to at least one repetitive nucleotide region, wherein the repetitive region is outside of the variable number tandem repeat (VNTR) domain of the nucleic acid, and wherein the repetitive nucleotide region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides, wherein the presence of the nucleic acid as part of a recombinant poxvirus improves the stability of the nucleic acid and/or the recombinant poxvirus through successive passaging as compared to a recombinant poxvirus encoding a MUC1 peptide without nucleotide substitution.

81. The nucleic acid of claim 80, wherein the repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

82. The nucleic acid of any one of claims 80-81 wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

83. The nucleic acid of any one of claims 80-82, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

84. The nucleic acid of claims any one of 80-83, wherein the nucleotide substitution is to at least 2, 3, 4, or 5 repetitive nucleotide regions of the nucleic acid.

85. The nucleic acid of any one of claims 80-84, wherein the nucleotide substitution is in at least 10, 15, 20, or 25 repetitive nucleotide regions of the nucleic acid.

86. The nucleic acid of any one of claims 80-85, wherein the nucleic acid further comprises a first modification to the VNTR domain, wherein the first modification comprises a shuffling of the order of the VNTR domain repeats.

87. The nucleic acid of any one of claims 80-86, wherein the nucleic acid further comprises a second modification to the VNTR domain, wherein the second modification comprises a codon optimization of the VNTR domain repeats.

88. The nucleic acid of any one of claims 80-87, wherein the nucleotide substitution does not modify the amino acid sequence of the MUC1 peptide.

89. The nucleic acid of any one of claims 80-88, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 4, 95% homologous to SEQ ID NO: 3, 95% homologous to SEQ ID NO: 5, or 95% homologous to SEQ ID NO:2 (336 MUC1).

90. The nucleic acid of any one of claims 80-89, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 2.

91. The nucleic acid of any one of claims 80-90, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 4.

92. The nucleic acid of any one of claims 80-91, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 3.

93. The nucleic acid of any one of claims 80-92, wherein the nucleic acid comprises SEQ ID NO: 4.

94. The nucleic acid of any one of claims 80-93, wherein the nucleic acid comprises SEQ ID NO: 3.

95. The nucleic acid of any one of claims 80-94, wherein the nucleic acid comprises SEQ ID NO:2.

96. A nucleic acid encoding a CEA peptide, the nucleic acid comprising a nucleotide substitution to at least one repetitive nucleotide region, wherein the repetitive region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T nucleotides, wherein the presence of the nucleic acid as part of a recombinant poxvirus improves the stability of the nucleic acid and/or the recombinant poxvirus through successive passaging as compared to a recombinant poxvirus encoding a CEA peptide without the substitution.

97. The nucleic acid of claim 96, wherein the repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

98. The nucleic acid of any one of claims 96-97, wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T nucleotides.

99. The nucleic acid of any one of claims 96-98, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

100. The nucleic acid of any one of claims 96-99, wherein the nucleotide substitution is to at least 2, 3, 4, 5, 8, or 10 repetitive nucleotide regions of the nucleic acid.

101. The nucleic acid of any one of claims 96-100, wherein the nucleotide substitution does not modify the amino acid sequence of the CEA peptide.

102. The nucleic acid of any one of claims 96-101, wherein the nucleic acid comprises SEQ ID NO: 14.

103. A nucleic acid encoding a B7-1 peptide, the nucleic acid comprising a nucleotide substitution to at least one repetitive nucleotide region, wherein the repetitive region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T nucleotides, wherein the presence of the nucleic acid as part of a recombinant poxvirus improves the stability of the nucleic acid and/or the recombinant poxvirus through successive passaging as compared to a recombinant poxvirus encoding a B7-1 peptide without the nucleotide substitution.

104. The nucleic acid of claim 103, wherein the repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

105. The nucleic acid of any one of claims 103-104, wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

106. The nucleic acid of any one of claims 103-105, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

107. The nucleic acid of any one of claims 103-106, wherein the nucleotide substitution is to at least 2, 3, 4, 5, 8, or 10 repetitive nucleotide regions of the nucleic acid.

108. The nucleic acid of any one of claims 103-107, wherein the nucleotide substitution does not modify the amino acid sequence of the B7-1 peptide.

109. The nucleic acid of any one of claims 103-108, wherein the nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 15.

110. The nucleic acid of any one of claims 103-109, wherein the nucleic acid comprises SEQ ID NO: 15.

111. The nucleic acid of any one of claims 103-108, wherein the nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 17.

112. The nucleic acid of any one of claims 103-109, wherein the nucleic acid comprises SEQ ID NO: 17.

113. A nucleic acid encoding an ICAM-1 peptide, the nucleic acid comprising a nucleotide substitution to at least one repetitive nucleotide region, wherein the repetitive region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T nucleotides, wherein the presence of the nucleic acid as part of a recombinant poxvirus improves the stability of the nucleic acid and/or the recombinant poxvirus through successive passaging as compared to a recombinant poxvirus encoding a ICAM-1 peptide without the nucleotide substitution.

114. The nucleic acid of claim 113, wherein the repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

115. The nucleic acid of any one of claims 113-114, wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

116. The nucleic acid of any one of claims 113-115, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

117. The nucleic acid of any one of claims 113-116, wherein the nucleotide substitution is to at least 2, 3, 4, 5, 8, or 10 repetitive nucleotide regions of the nucleic acid.

118. The nucleic acid of any one of claims 113-117, wherein the nucleotide substitution does not modify the amino acid sequence of the ICAM-1 peptide.

119. The nucleic acid of any one of claims 113-118, wherein the nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 18.

120. The nucleic acid of any one of claims 113-119, wherein the nucleic acid comprises SEQ ID NO: 18.

121. The nucleic acid of any one of claims 113-118, wherein the nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 20.

122. The nucleic acid of any one of claims 113-118, wherein the nucleic acid comprises SEQ ID NO: 20.

123. A nucleic acid encoding a LFA-3 peptide, the nucleic acid comprising a nucleotide substitution to at least one repetitive nucleotide region, wherein the repetitive region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides, wherein the presence of the nucleic acid as part of a recombinant poxvirus improves the stability of the nucleic acid and/or the recombinant poxvirus through successive passaging as compared to a recombinant poxvirus encoding a LFA-3 peptide without the nucleotide substitution.

124. The nucleic acid of claim 123, wherein the repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

125. The nucleic acid of any one of claims 123-124, wherein the repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

126. The nucleic acid of claims 123-125, wherein the repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

127. The nucleic acid of any one of claims 123-126, wherein the nucleotide substitution is to at least 2, 3, 4, 5, 8, or 10 repetitive nucleotide regions of the nucleic acid.
128. The nucleic acid of any one of claims 123-127, wherein the nucleotide substitution does not modify the amino acid sequence of the LFA-3 peptide.
129. The nucleic acid of any one of claims 123-128, wherein the nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 21.
130. The nucleic acid of any one of claims 123-128, wherein the nucleic acid comprises SEQ ID NO: 21.
131. The nucleic acid of any one of claims 123-128, wherein the nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 23.
132. The nucleic acid of any one of claims 123-128, wherein the nucleic acid comprises SEQ ID NO: 23.
133. An expression cassette comprising a promoter and the recombinant nucleic acid of any one of claims 77-132 operably linked to the promoter.
134. An immunogenic composition comprising the recombinant poxvirus of any one of claims 1-38, the nucleic acid of any one of claims 77-132, or the expression cassette of claim 133.
135. A host cell comprising the recombinant poxvirus of any one of claims 1-38, the nucleic acid of any one of claims 76-132, or the expression cassette of claim 133.
136. A vector comprising the nucleic acid of any one of claims 77-132 or the expression cassette of claim 133.
137. A method for generating a recombinant poxvirus having a MUC1 transgene that is stable through successive passaging of the recombinant poxvirus, the method comprising:
providing any one of the nucleic acids of claims 77-132 or the expression cassette of claim 133,
inserting the nucleic acid or the expression cassette into a recombinant poxvirus.
138. The method of claim 137, wherein the recombinant poxvirus is selected from an orthopoxvirus and an avipoxvirus.
139. The method of claim 138, wherein the orthopoxvirus is selected from vaccinia, MVA, MVA-BN, and derivatives of MVA-BN.
140. The method of any one of claims 137-139, wherein the recombinant poxvirus is stable through up to four passages.
141. The method of claim 137, wherein the avipoxvirus is a fowlpox virus.

142. The method of any one of claims 137 and 141, wherein the avipoxvirus is stable through at least 3 or 4 passages, or is stable through at least 7 passages.
143. A method for generating a recombinant poxvirus encoding a MUC1 peptide that is stable through successive passaging of the recombinant poxvirus, the method comprising:
- providing a first nucleic acid encoding a MUC1 protein, the MUC1 protein comprising at least two VNTR domains;
 - shuffling or rearranging the order of the at least two VNTR domain repeats;
 - optimizing the codons of the at least two VNTR domain repeats;
 - inserting the first nucleic acid sequence into the poxvirus to generate a recombinant poxvirus that is stable through successive passaging of the recombinant poxvirus.
144. The method of claim 143, wherein the substitution does not modify the amino acid sequence of the MUC1 peptide.
145. The method of any one of claims 143-144, wherein the first nucleic acid is at least 95% homologous to SEQ ID NO:2, 95% homologous to SEQ ID NO: 4, 95% homologous to SEQ ID NO: 3, or 95% homologous to SEQ ID NO: 5.
146. The method of any one of claims 143-145, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 2.
147. The method of any one of claims 143-145, wherein the nucleic acid is at least 95% homologous to SEQ ID NO: 3.
148. The method of any one of claims 143-145, wherein the nucleic acid comprises SEQ ID NO: 2.
149. The method of any one of claims 143-145, wherein the nucleic acid comprises SEQ ID NO: 5.
150. The method of any one of claims 143-149, wherein the method further comprises substituting at least one nucleotide in a repetitive nucleotide region of a second nucleic acid encoding a CEA peptide, wherein the repetitive nucleotide region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides; and inserting the second nucleic acid in the recombinant poxvirus.
151. The method of claim 150, wherein the repetitive region of the second nucleic acid is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

152. The method of any one of claims 150-151, wherein the repetitive nucleotide region of the second nucleic acid is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

153. The method of any one of claims 150-152, wherein the repetitive region of the second nucleic acid is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

154. The method of any one of claims 150-153, wherein the substitution is to at least 2, 3, 4, 5, or 10 repetitive nucleotide regions of the second nucleic acid.

155. The method of any one of claims 150-154, wherein the second nucleic acid comprises SEQ ID NO:14.

156. The method of any one of claims 143-155, wherein the method further comprises substituting at least one nucleotide in a repetitive nucleotide region of a third nucleic acid encoding a costimulatory molecule selected from B7-1, ICAM-1, and/or LFA-3, CEA, wherein the repetitive nucleotide region is defined as: (i) three or more consecutively repeated nucleotides, (ii) three or more consecutive G or C nucleotides, and/or (iii) three or more consecutive T or C nucleotides; and inserting the third nucleic acid in the recombinant poxvirus.

157. The method of claim 156, wherein the costimulatory molecule repetitive region is further defined as (i) three or more consecutive G nucleotides, (ii) three or more consecutive C nucleotides, and/or (iii) three or more consecutive T nucleotides.

158. The method of any one of claims 156-157, wherein the costimulatory molecule repetitive nucleotide region is further defined as (i) four or more consecutively repeated nucleotides, (ii) four or more consecutive G or C nucleotides, and/or (iii) four or more consecutive T or C nucleotides.

159. The method of any one of claims 156-158, wherein the costimulatory molecule repetitive region is further defined as (i) four or more consecutive G nucleotides, (ii) four or more consecutive C nucleotides, and/or (iii) four or more consecutive T nucleotides.

160. The method of any one of claims 156-159, wherein the substitution is to at least 2, 3, 4, 5, or 10 repetitive nucleotide regions of the costimulatory molecule nucleic acid.

161. The method of any one of claims 156-160, wherein the nucleic acid encoding the costimulatory molecule is selected from B7-1, ICAM-1, LFA-3, and combinations thereof.

162. The method of any one of claims 156-160, wherein the nucleic acid encoding the B7.1 costimulatory molecule is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 15 or SEQ ID NO: 17.
163. The method of any one of claims 156-162, wherein the nucleic acid encoding the B7.1 costimulatory molecule comprises SEQ ID NO: 15 or SEQ ID NO: 17.
164. The method of any one of claims 156-163, wherein the nucleic acid encoding the ICAM-1 costimulatory molecule is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 18 or SEQ ID NO: 20.
165. The method of any one of claims 156-164, wherein the nucleic acid encoding the ICAM-1 costimulatory molecule comprises SEQ ID NO: 18 or SEQ ID NO: 20.
166. The method of any one of claims 156-164, wherein the nucleic acid encoding the LFA-3 costimulatory molecule is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NO: 21 or SEQ ID NO: 23.
167. The method of any one of claims 156-164, wherein the nucleic acid encoding the LFA-3 costimulatory molecule comprises SEQ ID NO: 21 or SEQ ID NO: 23.
168. The method of any one of claims 143-167, wherein the poxvirus is an orthopoxvirus.
169. The method of claim 168, wherein the orthopoxvirus is a vaccinia virus.
170. The method of claim 169, wherein the vaccinia virus is a modified vaccinia Ankara (MVA) virus.
171. The method of claim 170, wherein the MVA is MVA-BN or a derivative or MVA-BN.
172. The method of any one of claims 168-171, wherein the poxvirus is stable through at least passage 3 or passage 4.
173. The method of any of claims 143-167, wherein the poxvirus is an avipoxvirus.
174. The method of claim 173, wherein the avipoxvirus is a fowlpox virus.
175. The method of any one of claims 173-174, wherein the poxvirus is stable through at least Passage 4.
176. Use of a) a nucleic acid of any one of claims 77-132, b) an expression cassette of claim 133, c) a composition of claim 134, d) a host cell according to claim 135, or e) a vector according to claim 136 in a method for generating a recombinant poxvirus that is stable through successive passaging of the poxvirus.

177. The use of claim 176, wherein the poxvirus is selected from a vaccinia virus, MVA, MVA-BN, or a derivative of MVA-BN, and wherein the poxvirus is stable through passage 4.

178. The use of claim 176, wherein the poxvirus is selected from an avixpoxvirus and a fowlpox virus, and wherein the poxvirus is stable through at least passage 3 or passage 4.

179. Use of a) a recombinant poxvirus according to any one of claims 1-38, b) a nucleic acid of any one of claims 77-132, b) an expression cassette of claim 133, c) a composition of claim 134, d) a host cell according to claim 135, or e) a vector according to claim 136 in the preparation of a medicament preferably a vaccine.

180. A recombinant poxvirus according to any one of claims 1-38, b) a nucleic acid of any one of claims 77-132, b) an expression cassette of claim 133, c) a composition of claim 134, d) a host cell according to claim 135, or e) a vector according to claim 136 for use as a medicament preferably a vaccine.

181. A recombinant poxvirus according to any one of claims 1-38 and 180, b) a composition of claim 134, or c) a vector according to claim 136 for use in a heterologous prime-boost dose regimen of a vaccine.

182. The recombinant poxvirus, the composition or vector for use of claim 181, wherein the heterologous prime-boost dosing regimen comprises:

a) one or more priming doses of a vaccinia virus, the vaccinia virus including a nucleic acid according to any one of claims 77-132; and

b) one or more boosting doses of an fowlpox virus including a nucleic acid according to any one of claims 77-132.

183. The recombinant poxvirus, the composition or vector for use of claim 182, wherein the vaccinia virus is selected from a modified vaccinia virus Ankara (MVA), MVA-BN, and derivatives of MVA-BN.

184. A nucleic acid of any one of claims 77-132, b) an expression cassette of claim 133, c) a composition of claim 134, d) a host cell according to claim 135, or e) a vector according to claim 136 for use in generating a recombinant poxvirus that is stable through successive passaging.

185. A method for generating a recombinant poxvirus that is stable through successive passaging of the recombinant poxvirus, the method comprising:

a) providing a first nucleic acid encoding a MUC1 peptide having at least two Variable N-Terminal Repeat (VNTR) domains, wherein the arrangement of the at least two VNTR domains are shuffled, and b) the at least two VNTR domains are codon optimized; and

b) providing a second nucleic acid encoding a CEA peptide, wherein the second nucleic acid comprises at least one nucleotide substitution in at least one repetitive nucleotide region of the second nucleic acid, wherein the at least one repetitive nucleotide region is defined as a) three more consecutively repeated G or C nucleotides and/or b) three or more consecutively repeated T nucleotides.

186. The method of claim 185, wherein the first nucleic acid comprises at least three VNTR domains.

187. The method of any one of claims 185-186, wherein the order of the at least two VNTR domains are shuffled as compared to SEQ ID NO: 6.

188. The method of any one of claims 185-187, wherein the first nucleic acid sequence comprises a nucleic acid sequence having at least 95%, 96%, 97%, or 98% identity to SEQ ID NO:2.

189. The method of any one of claims 185-188, wherein the first nucleic acid sequence comprises SEQ ID NO: 2.

190. The method of any one of claims 185-189, wherein the second nucleic acid further comprises at least one nucleotide substitution in at least one repetitive nucleotide region of the second nucleic acid, wherein the at least one repetitive nucleotide region is defined as a) three more consecutively repeated G or C nucleotides and/or b) three or more consecutively repeated T nucleotides.

191. The method of any one of claims 185-190, wherein the second nucleic acid comprises at least one substitution to at least 2, 3, 4, 5, or 10 of the repetitive nucleotide regions of the second nucleic acid.

192. The method of any one of claims 185-191, wherein the second nucleic acid comprises at least one nucleotide substitution in at least 10, at least 12, at least 15, and/or at least 19 repetitive nucleotide regions of the second nucleic acid

193. The method of any one of claims 185-192, wherein the second nucleic acid comprises SEQ ID NO: 14.

194. The method of any one of claims 185-193, wherein the recombinant poxvirus is selected from a vaccinia virus, MVA, MVA-BN, and derivatives of MVA-BN and is stable through at least passage 3 or passage 4.

195. The method of any one of claims 185-193, wherein the recombinant poxvirus is selected from an avipoxvirus and a fowlpox virus and is stable through at least passage 3 or passage 4.

196. The method of any one of claims 185-193, wherein the recombinant poxvirus is selected from an avipoxvirus and a fowlpox virus and is stable through at least passage 7.