

COMPOSITIONS AND METHODS FOR ENHANCING THE STABILITY OF TRANSGENES IN POXVIRUSES

FIELD OF THE INVENTION

[001] The present invention relates to recombinant poxviruses and immunogenic compositions thereof. Also disclosed are immunogenic compositions 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 pox viruses 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)).

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 present techniques, difficulties involving large scale production, impurities, and other issues have been successfully overcome.

BRIEF SUMMARY OF THE INVENTION

[013a] Aspects of the invention are defined in the independent claims. Some optional features of the invention are defined in the dependent claims.

[014] It was determined 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 disclosure 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 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 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 disclosure 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 techniques relate 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 or 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 present techniques 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 techniques. These may 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 exemplary, non-limiting 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. 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. 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 disclosure. 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 disclosure. 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 disclosure. 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 disclosure. 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 disclosure. 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.

[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 disclosure, 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

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 disclosure 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 disclosure 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 disclosure 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 exemplary 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

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 disclosure 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.

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 techniques, 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 techniques, 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 disclosure 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 disclosure 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 disclosure 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 disclosure 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 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 exemplary technique, 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 techniques, 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
GTTACGGGTT	CTGGTCATGC	AAGCTCTACC	CCAGGTGGAG	AAAAGGAGAC	TTCGGCTACC	120
CAGAGAAGTT	CAGTGCCAG	CTCTACTGAG	AAGAATGCTG	TGAGTATGAC	AAGCTCCGTA	180
CTCTCCAGCC	<u>ACAGCCCCGG</u>	<u>TTCAGGCTCC</u>	<u>TCCACCACTC</u>	<u>AGGGACAGGA</u>	<u>TGTCACCTCTG</u>	240
<u>GCCCCGGCCA</u>	<u>CGGAACCAGC</u>	<u>TTCAGGTTCA</u>	<u>GCTGCCTTGT</u>	<u>GGGGACAGGA</u>	<u>TGTCACCTCTG</u>	300
GTACCAGTTA	CTAGACCAGC	TTTAGGTTAGC	ACAGCACCTC	CTGCTCATGG	AGTAACTAGT	360
GCTCCTGATA	CTCGTCCAGC	TCCTGGCAGT	ACTGCACCAC	CGGCACATGG	CGTAACATCA	420
GCACCTGATA	CAAGACCTGC	ACCTGGATCT	ACAGCGCCGC	CTGCGCACGG	AGTGACATCG	480
GCGCCCGATA	CGCGCCCCGC	TCCCGGTAGC	ACCGCACCGC	CCGCCCCACGG	TGTTACAAGT	540
GCACCCGATA	CCCGGCCCGC	ACCCGGAAGT	ACCGCTCCAC	CTGCACACGG	GGTCACAAGC	600
GCGCCAGACA	CTCGACCTGC	GCCAGGGTCG	ACTGCCCTC	CGGCGCATGG	TGTGACCTCA	660
GCTCCTGACA	CAAGGCCAGC	CCCAGCTAGC	ACTCTGGTGC	ACAACGGCAC	<u>CTCTGCCAGG</u>	720
GCTACCACAA	<u>CCCCAGCCAG</u>	CAAGAGCACT	CCATTCTCAA	<u>TTCCCAGCCA</u>	<u>CCA</u> CTCTGAT	780

ACTCCTACCA	<u>CCCTTGCCAG</u>	CCATAGCACC	AAGACTGATG	CCAGTAGCAC	TCACCATAGC	840
ACGGTACCTC	<u>CTCTCACCTC</u>	<u>CTCCAATCAC</u>	AGCACTTCTC	<u>CCCAGTTGTC</u>	TACTGGGGTC	900
<u>TCTTTCTTTT</u>	<u>TCCTGTCTTT</u>	<u>TCACATTTCA</u>	<u>AACCTCCAGT</u>	<u>TTAATTCCTC</u>	<u>TCTGGAAGAT</u>	960
CCCAGCACCG	ACTACTACCA	AGAGCTGCAG	AGAGACATTT	<u>CTGAAATGTT</u>	<u>TTTGCAGATT</u>	1020
TATAAACAAAG	<u>GGGGTTTTCT</u>	<u>GGGCCTCTCC</u>	AATATTAAGT	TCAGGCCAGG	<u>ATCTGTGGTG</u>	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
<u>GTGAGTGATG</u>	<u>TGCCATTTCC</u>	<u>TTTCTCTGCC</u>	<u>CAGTCTGGGG</u>	<u>CTGGGGTGCC</u>	<u>AGGCTGGGGC</u>	1260
<u>ATCGCGCTGC</u>	<u>TGGTGCTGGT</u>	<u>CTGTGTTCTG</u>	<u>GTTGCGCTGG</u>	<u>CCATTGTCTA</u>	<u>TCTCATTGCC</u>	1320
TTGGCTGTCT	<u>GTCAGTGCCG</u>	<u>CCGAAAAGAAC</u>	<u>TACGGGCAGC</u>	TGGACATCTT	TCCAGCCCCG	1380
GATACCTACC	<u>ATCCTATGAG</u>	<u>CGAGTACCCC</u>	<u>ACCTACCACA</u>	<u>CCCATGGGCG</u>	<u>CTATGTGCCC</u>	1440
<u>CCTAGCAGTA</u>	<u>CCGATCGTAG</u>	<u>CCCCTATGAG</u>	<u>AAGGTTTCTG</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 techniques 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 exemplary embodiment, there is a novel MUC1 nucleic 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 MUC1 nucleic 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 MUC1 nucleic 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 MUC1 nucleic acid can comprise at least one substitution in those regions outside of the VNTR regions that are more prone to mutate

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

It is understood by the present disclosure 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 disclosure 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 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 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 disclosure, 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 exemplary embodiments, the present techniques include 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 disclosure, 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 techniques, 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 disclosure 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 techniques 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

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 techniques 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 disclosure that the nucleic acids (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 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.

[0127] In various embodiments, the disclosure 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 disclosure, 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).

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 disclosure 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 disclosure 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 disclosure, 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

11. The recombinant poxvirus of any one of claims 9 – 10, 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.
12. The recombinant poxvirus of any one of claims 9 – 11, wherein the second nucleic acid comprises SEQ ID NO: 14.
13. The recombinant poxvirus of any one of the preceding claims, wherein the recombinant poxvirus is selected from an orthopoxvirus and an avipoxvirus.
14. The recombinant poxvirus of claim 13, wherein the orthopoxvirus is a vaccinia virus.
15. The recombinant poxvirus of claim 14, wherein the vaccinia virus is a modified vaccinia virus Ankara (MVA).
16. The recombinant poxvirus of claim 15, wherein the MVA is MVA-BN or a derivative of MVA-BN.
17. The recombinant poxvirus of any one of the preceding claims, wherein the recombinant poxvirus is stable through at least Passage 3 and/or Passage 4.
18. The recombinant poxvirus of claim 17, wherein the avipoxvirus is a fowlpox virus.
19. The recombinant poxvirus of any one of the preceding claims, 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.
20. The recombinant poxvirus of any one of the preceding claims, wherein the poxvirus further comprises a nucleic acid encoding one or more co-stimulatory molecules.
21. The recombinant poxvirus of claim 20, wherein the one or more co-stimulatory molecules are selected from B7-1, ICAM-1, LFA-3, and combinations thereof.

22. The recombinant poxvirus of claim 21, wherein the B7-1 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 15, 16, or 17.

23. The recombinant poxvirus of any one of claims 21-22, wherein the B7-1 nucleic acid comprises SEQ ID NO: 15, 16, or 17.

24. The recombinant poxvirus of claim 21, wherein the ICAM-1 nucleic acid is at least 80%, 85%, 90%, or 95% homologous to SEQ ID NOs: 18, 19, or 20.

25. The recombinant poxvirus of any one of claims 21 and 24, wherein the ICAM-1 nucleic acid comprises SEQ ID NO: 18, 19, or 20.

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

27. The recombinant poxvirus of claim 26, wherein the LFA-3 nucleic acid comprises SEQ ID NO: 21, 22, or 23.

28. An immunogenic composition comprising the recombinant poxvirus of any one of claims 1-27.

29. A recombinant poxvirus according to any one of claims 1 – 27 for use in a heterologous prime-boost dose regimen of a vaccine.

30. The recombinant poxvirus, the composition or vector for use of claim 29, wherein the heterologous prime-boost dosing regimen comprises:

- a) one or more priming doses of a vaccinia virus; and
- b) one or more boosting doses of a fowlpox virus.