HUMAN CYTOMEGALOVIRUS COMPRISING EXOGENOUS ANTIGENS

CROSS REFERENCE TO RELATED APPLICATION

The present application claims priority to U.S. Provisional Application No. 62/025,348, filed July 16, 2014, entitled HUMAN CYTOMEGALOVIRUS COMPRISING EXOGENOUS ANTIGENS, the disclosure of which is hereby incorporated by reference in its entirety.

FIELD

Generally, the field involves vaccine platforms. More specifically, the field involves recombinant human cytomegalovirus vectors expressing exogenous antigen.

BACKGROUND

Animal experiments have demonstrated that cytomegalovirus (CMV)-vectored vaccines are unique in that they: a) induce and maintain high frequencies of extralymphoid T cell responses (so called effector memory T cells); b) super-infect CMV-positive hosts; and c) maintain immunogenicity even when rendered deficient in host-to-host spread. Furthermore, experiments in animal models have shown that vaccine vectors derived from animal CMVs induce a protective immune response against infectious diseases and cancer (US 20080199493; US 20100142823; US 20130136768; and US 20140141038; all of which are incorporated by reference herein). Particularly striking is the finding that a rhesus CMV (RhCMV)-vectored simian immunodeficiency virus (SIV)-vaccine was able to not only prevent AIDS in non-human primates, but ultimately cure these animals from SIV (Hansen SG *et al.*, Nature **502**, 100-104 (2013); incorporated by reference herein).

It is important to use an attenuated strain in the development of a cytomegalovirus vaccine because an unattenuated strain could spread from host to host and potentially be pathologic at least in immunocompromised individuals. Previously, attenuated human CMV (HCMV) strains have failed to a) establish latent infection (Plotkin SA and Huang ES, *J Infect Dis* **152**, 395-397 (1985); incorporated by reference herein); b) induce long-lasting immunity (Jacobson MA *et al.*, *J Clin Virol* **35**, 332-337 (2006); incorporated by reference herein); c)

reinfect the significant proportion of the population that has been previously naturally infected with CMV (Heineman TC *et al.*, *J Infect Dis* **193**, 1350-1360 (2006); incorporated by reference herein); or d) produce persistent infections (WO2013/036465; incorporated by reference herein.) Furthermore, clinical strains of HCMV genomes are highly unstable in vitro when grown in fibroblasts, resulting in fibroblast adaptations such as deletion of UL131A.

The impact of such adaptations to tissue culture for the ability to perform vector functions in vivo is mostly unknown. In addition to the need for attenuations to be stable in vitro and in vivo, it is important that these vectors can be manufactured with reproducible results. The most stable attenuation strategy is gene deletion. However, this generally requires the generation of complementing cell lines which is difficult to achieve for primary cells used to grow cytomegalovirus.

SUMMARY

Disclosed herein are severely attenuated, spread-deficient (*i.e.*, deficient in cell to cell spread) vectors derived from HCMV-TR3, which is a genetically modified version of the HCMV TR strain. The disclosed vectors establish and maintain persistent infections, induce and maintain effector memory T cells against heterologous antigens, and re-infect CMV-seropositive hosts. Said vectors comprise heterologous antigens such as non-CMV pathogen specific antigens or tumor antigens.

Specifically, TR3 was engineered to be ganciclovir-sensitive. In one example, this is due to the addition of an active UL97 gene (which was mutated in the original clinical isolate of TR3). TR3 was further engineered to include active US2, US3, US6, and US7 genes which were removed during BAC cloning of the original clinical isolate of TR3. Additional versions of TR3 include a deleterious (*i.e.*, inactivating) mutation in the pp71-encoding UL82 gene – which can be termed TR3Δpp71 or, alternatively TR3ΔUL82 herein.

In further examples of the vectors, the expression of a gene encoding a heterologous antigen can be driven by the UL82 promoter or another viral promoter such as the UL7, UL38, UL45, or US13 promoter. In still further examples, multiple genes encoding heterologous antigens can be inserted in place of UL82 and another viral gene such as UL7,

UL38, UL45, or US13 such that the viral gene promoter drives expression of the heterologous antigen gene.

Also disclosed herein is a method of producing an HCMV lacking a functional pp71 protein (encoded by the UL82 gene). The method involves infecting a cell with the HCMV lacking a functional pp71 protein, wherein the cell contains an siRNA that silences the DAXX gene. In other embodiments, the method involves infecting a cell with the HCMV lacking a functional pp71 protein, wherein expression of the DAXX gene in the cell is downregulated at the protein or RNA level by other techniques known in the art, for example by RNA interference (e.g., microRNA targeting and short hairpin RNA (shRNA) targeting), ribozyme cleavage, regulated expression by a conditional or inducible promoter, expression of DAXX binding proteins, or targeting DAXX or DAXX protein complexes for ubiquitination and degradation. Using these methods, the HCMV is produced efficiently without complementation. The cell can be any cell, including a human fibroblast.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some of the drawings herein are better understood when presented in color, which is not available in patent application publications. However, Applicants consider the color drawings to be part of the original disclosure and reserve the right to present color versions of the drawings herein in later proceedings.

Figures 1A and **1B** collectively show that HCMV TR is superior in establishing latency and in reactivating from latency (+G-CSF) compared to other HCMV strains. **Figure 1A** is a map of the genome organization of the HCMV clones used in **Figure 1B**. HCMV genomes are flanked by terminal repeats (TRL and TRS as indicated) and internal repeats (IRS) that separate the unique long (UL) and unique short (US) regions. The location of the BAC cassette in each construct is indicated by the region indicated as B. The US region of HCMV TR lacks US2-7 due to insertion of the BAC-cassette. TRΔ4 lacks the genes UL128-UL150 in addition to lacking US2-7. The UL131A gene is deficient in AD169 but repaired in AD169 BAD UL131A (Wang and Schenk, 2005 *infra*). Toledo has an inversion of the UL133-128 region with a deletion in UL128 (Murphy *et al.*, 2003 *infra*). **Figure 1B** is a plot summarizing the results of NOD/SCID/IL2Rγ-null (NSG) mice engrafted with human CD34+ stem cells and inoculated intraperitoneally with human fibroblasts infected with the indicated HCMV

strains. Four weeks after infection, human hematopoietic stem cells were mobilized by granulocyte colony-stimulating factor (G-CSF) treatment, and the viral load was measured in the liver by quantitative PCR.

Figure 2 is a graphical representation of the HCMV-TR3 genome showing alterations to the open reading frames (ORFs) present in the original HCMV TR strain. To confer ganciclovir sensitivity, UL97 of HCMV TR was replaced with that of HCMV AD169. The BAC cassette is flanked by loxP sites, and, after cre-mediated self-excision, a single loxP site remains in the genome. Since the HCMV-TR BAC lacks US2-7, the corresponding genes from HCMV AD169 were inserted. The terminal (ab and c'a) repeats and internal repeats (b'a'c) are shown.

Figure 3 is a plot showing that HCMV-TR3, but not HCMV-TR, is sensitive to ganciclovir (GCV). Growth-arrested human fetal fibroblast MRC-5 cells were infected with HCMV TR3, HCMV TB40E, and original HCMV TR (MOI of 1 PFU/cell) or mock infected. Where indicated, cells were treated with increasing concentrations of GCV 90 min after infection until an extensive viral cytopathic effect was observed in the untreated control (4 days post-infection). Supernatants of cell cultures were then assayed for infectivity by standard plaque reduction assay on MRC-5 cells. The number of plaques was plotted as a function of drug concentration, and the IC_{50} was determined. Values are the means of two independent determinations.

Figures 4A and **4B** show that HCMV-TR3 surprisingly maintains the ability to infect endothelial cells and maintains genome stability after multiple passaging. **Figure 4A** is an image of a gel showing the following: HCMV-TR3 BAC was reconstituted on MRC-5 cells and then passaged 20 times *in vitro* on primary human fibroblasts. At passage 1, 5, 10, 15, and 20, viral DNA was extracted from infected cells and subjected to restriction digestion analysis and PCR sequencing of the UL128-131 region, a region that is frequently mutated as a result of multiple passaging (Dargan *et al.*, 2010, *infra*). **Figure 4B** is a plot showing the infectivity of TR3 in human umbilical vein endothelial cells (HUVECs) after multiple passages on MRC-5 cells. A purified stock of virus was made at passage 10 and used to infect HUVECs at MOI=0.5. At the same time, HUVECs were also infected with the HCMV lab adapted strain AD169 as control. Supernatants and cells were harvested at 5, 10, 15, and 20 days post infection (pi) and titrated by plaque assay on MRC-5 cells. The increase in titers over time

indicates that HCMV TR3 was able to grow on HUVECs, consistent with an intact UL131A-128 region, whereas HCMV AD169 does not grow.

Figure 5 is a plot showing that the presence of UL128-131 does not reduce the yield of cell-free HCMV-TR3. A multiple-step growth curve analysis was conducted using MRC-5 cells infected at MOI 0.01 with HCMV-TR3 and a strain identical to TR3 but with the UL128-131 deleted (HCMVΔUL128-131). Titers of infected cells and supernatants were measured at 2, 5, 10, 15 and 20 days post infection by standard plaque assay on MRC-5 cells.

Figure 6A is a set of two plots showing the results when SIVgag under control of the EF1α promoter was inserted into the HCMV-TR3 genome using BAC mutagenesis as described in Hansen SG *et al.*, *Nat Med* **15**, 293-299 (2009) (incorporated by reference herein). Rhesus macaques (RM) sero-positive for CMV were inoculated with 10^5 plaque-forming units (PFU) of HCMV-TR expressing SIVgag. Shown is the % memory T cells in peripheral blood mononuclear cells (PMBC) responding to HCMV lysate (diamonds) or over-lapping SIVgag (squares) peptides. Note the absence of T cells to the canonical CM9 peptide (circles), indicating that the T cell response induced by HCMV is different from that of other vectors as described for RhCMV (Hansen *et al.*, *Science* 2013 *infra*). The plot on the left shows $CD4^+$ T cell responses. The plot on the right shows $CD8^+$ T cell responses.

Figure 6B is a set of two plots showing the HIVgag-specific T cell responses in RM inoculated with HCMV expressing HIVgag under control of the UL78 promoter with UL128-131 deleted (ΔUL128-131 HCMVgag) or HCMV expressing HIVgag under control of the UL82 promoter with UL128-131 intact (Δpp71 HCMVgag). When 10^6 PFU of the ΔUL128-131 vector were inoculated into RM, no $CD4^+$ or $CD8^+$ T cell response to HIVgag was observed. In contrast, HIVgag-specific T cell responses were observed with Δpp71 HCMVgag vectors. The plot on the left show $CD4^+$ T cell responses, the plot on the right shows $CD8^+$ T cell responses.

Figure 7A is a drawing illustrating how, during infection with wildtype HCMV, the tegument protein pp71 degrades the cellular corepressor DAXX. In the absence of pp71, DAXX represses viral gene expression and thus lytic replication. However, viral gene expression can proceed normally even in the absence of pp71 when DAXX mRNA is eliminated by gene knockdown with DAXX-specific siRNA.

Figure 7B is a plot of MRC-5 cells transfected with DAXX-specific siRNA and infected 24 hours (h) post-transfection with TR3 and TR3Δpp71HIVgag at MOI=0.05. At the indicated times post-infection, cells and supernatants were harvested separately and titered on complementing cells expressing pp71.

Figures 8A and **8B** are plots showing that HCMVTR3ΔUL82 (Δpp71) establishes latency in humanized mice but is deficient in its ability to reactivate and disseminate**.** For both plots, NOD/SCID/IL2Rγ-null (NSG) mice engrafted with CD34+ stem cells were inoculated intraperitoneally with fibroblasts infected with TR3 or TR3ΔUL82 virus. Four weeks post-infection, human hematopoietic stem cells were mobilized by G-CSF treatment, and the viral load was measured in bone marrow (TR3, **Figure 8A**) and liver (TR3ΔUL82, **Figure 8B**) by quantitative PCR.

Figure 9 is a set of plots showing that pp71-deleted HCMV-TR3 expressing HIVgag maintains the ability to induce HIVgag-specific effector memory T cells in non-human primates**.** HCMV expressing HIVgag but lacking pp71 was constructed by replacing the UL82(pp71) gene with HIVgag. The resulting virus was recovered using DAXX siRNA. 10^6 or 10^5 PFU of the resulting virus was inoculated subcutaneously into RM, and the T cell response to HIVgag was determined at the indicated days by intracellular cytokine staining. Shown is the percentage of $CD4^+$ (left) and $CD8^+$ (center) memory T cells in peripheral blood mononuclear cells (PMBC) responding to over-lapping HIVgag peptides. The right panel shows that the responding T cells display effector memory phenotype.

Figure 10A is a set of six plots showing the results of dual RhCMV vectors expressing both SIVenv and SIVpol. The dual expression vectors were constructed by first replacing Rh110 (the RhCMV homologue of pp71) with SIVenv. Next, the homologs of HCMV genes UL7 (Rh19), UL78 (Rh107) or US13 (Rh191) were replaced with SIVpol. The resulting vectors were recovered in pp71-expressing rhesus fibroblasts. $5x10^6$ PFU of each vector was inoculated into two RM each (one RM is shown as solid line, the other RM is shown as stippled line). The $CD4^+$ and $CD8^+$ T cell response was measured in PBMC at the indicated days using overlapping 15mer peptides corresponding to either SIVpol or SIVenv. The percent SIV-specific T cells within the T cell memory pool is shown.

Figure 10B is an image of an SDS-PAGE gel showing the results when MRC-5 cells were mock-infected or infected with TR3ΔUL7HIVgag, TR3ΔUL45HIVgag, or

TR3ΔUL78HIVgag at MOI 0.5. Protein extracts were prepared 96 hours post-infection (hpi). 20 micrograms of proteins were separated on 10% SDS-PAGE, and the immunoblot was decorated with an anti-Gag (p24) antibody.

Figure 11 is a set of two plots showing results with SIVgag under control of the EF1α promoter. SIVgag was inserted into the HCMV-TR3 genome using BAC mutagenesis as described in Hansen SG *et al.*, *Nat Med* **15**, 293-299 (2009) (incorporated by reference herein). Rhesus macaques (RM) sero-positive for CMV were inoculated with 10^5 plaque-forming units (PFU) of HCMV-TR3 expressing SIVgag. Shown is the % CD4+ (left panel) and % CD8+ (right panel) T cells in peripheral blood mononuclear cells (PMBC) responding to over-lapping SIVgag peptides. Note that the plot shows a stable immune response for two rhesus monkeys (Rh31017, Rh31219) beyond 378 days post inoculation.

Figure 12 plots the T cell immune response of two RM inoculated with the TR3ΔUL78 HCMV/HIVgag ΔUL128-130. Unlike constructs that included deletion of UL131A, limiting the deletion to UL128-130 results in sustained CD4+ and CD8+ T cell responses.

Figure 13 is a plot comparing the growth kinetics of wild-type TR3 (squares) vs. ΔUL82(pp71)HIVgag in the presence (circles) or absence (diamonds) of DAXX siRNA over a range of infectious particles per cell. The growth defect becomes visible at clinically relevant low MOI, where MRC-5 cells transfected with DAXX-specific siRNA and infected 24 h post-transfection with TR3 and TR3Δpp71HIVgag are functionally complemented by siRNA or fail to replicate in the absence of DAXX siRNA. The lack of replication at low MOI indicates tha the virus is deficient in cell to cell spread. At the indicated times post-infection, supernatants were harvested and titered under pp71 complementing conditions (DAXX siRNA transfected MRC-5 cells).

Figure 14 is a set of three graphs demonstrating that HCMVTR3ΔUL82 (Δpp71) establishes latency in humanized mice but is deficient in its ability to reactivate and disseminate**.** NOD/SCID/IL2Rγ-null (NSG) mice engrafted with CD34+ stem cells were inoculated intra-peritoneally with fibroblasts infected with TR3, TR3ΔUL82, or TR3ΔUL82ΔUL128-130 virus. Four weeks post-infection, human hematopoietic stem cells were mobilized by G-CSF treatment and the viral load was measured in bone marrow (upper left panel), liver (upper right panel), and spleen (bottom panel). The relative virus copy number as a function of total micrograms of DNA are plotted based on quantitative PCR.

Values in the absence of granulocyte colony stimulating factor (G-CSF) represent the latent viral load and values after G-CSF stimulation represent the reactivation of virus emerging from latency. Constructs deleted for pp71 establish latent infection but fail to respond to G-CSF stimulation as measured by copies of virus genomic DNA.

Figure 15 is a set of three plots characterizing the immune response of three RM inoculated with the TR3/HCMV Δpp71(HIVgag) construct. The vector was grown and titered in the presence of siRNA and concentrated for subcutaneous inoculation. Shown is the percentage of $CD4^+$ (left panel) and $CD8^+$ (middle panel) memory T cells in peripheral blood mononuclear cells (PMBC) responding to over-lapping HIVgag peptides. Responses to different doses of the construct are graphed to 294 days post inoculum. The right panel demonstrates the CD8+ response of the Δpp71(HIVgag) TR3/HCMV to be consistent with the T-effector memory phenotype.

Figure 16 graphically depicts the sequence alignment of HCMV/TR3 ΔUL82(pp71)HIVgag through passage 9 compared to the BAC clone sequence. The open reading frames (ORFs) are depicted as arrows, where the self-excising BAC is depicted with white arrows, the viral ORFs are depicted with grey arrows, and the HIVgag insert replacing the UL82 ORF is depicted with black arrows. Internal and terminal repeats are depicted with grey ovals. No significant polymorphisms were observed LOD 1%.

Figures 17a and **17b** confirm the gag insert expression and homogeneity over several infectious cycles. **Figure 17a** depicts a composite Western blot confirming the absence of pp71 protein expression in the ΔUL82(pp71) constructs and the presence of HIVgag(p24) expression. A positive control for HCMV expression (pp28) and a loading control to beta-Actin are included. **Figure 17b** shows the sequence of the gag insert is stable through these early passages with no polymorphisms detected by Sanger sequencing.

Figure 18 is a plot showing an example of how alternative insertion sites and promoters can affect insert stability. In this example, the EF1a promoter driving the SIVgag insert has been placed into the UL36 locus. This construct shows the emergence of polymorphisms above the background level. In this case, the emergence of a G > T substitution generates a stop codon, thereby truncating the vectored antigen.

SEQUENCE LISTING

SEQ ID NO: 1 is the nucleic acid sequence of HCMV TR3ΔUL82 BAC

SEQ ID NO: 2 is the nucleic acid sequence of the sense strand of an siRNA that silences DAXX.

SEQ ID NO: 3 is the nucleic acid sequence of the antisense strand of an siRNA that silences DAXX.

Homo sapiens DAXX mRNA includes a number of splice variants. Examples of the splice variants include the following GenBank entries: AB015051; CR457085; AF006041; NM_001254717.1; NM_001350; NM_001141969; NM_001141970; HQ436529; HQ436528; all of which are incorporated by reference herein.

DETAILED DESCRIPTION

Terms:

As used herein, the term “antigen” refers to a substance, typically a protein, which is capable of inducing an immune response in a subject. The term also refers to proteins that are immunologically active in the sense that once administered to a subject (either directly or by administering to the subject a nucleotide sequence or vector that encodes the protein) is able to evoke an immune response of the humoral and/or cellular type directed against that protein.

As used herein, the terms “nucleotide sequences” and “nucleic acid sequences” refer to deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences, including, without limitation, messenger RNA (mRNA), DNA/RNA hybrids, or synthetic nucleic acids. The nucleic acid can be single-stranded, or partially or completely double-stranded (duplex). Duplex nucleic acids can be homoduplex or heteroduplex.

As used herein, the term "small interfering RNA" ("siRNA") (also referred to in the art as "short interfering RNAs") refers to an RNA agent, preferably a double-stranded agent, of about 10-50 nucleotides in length (the term "nucleotides" including nucleotide analogs), preferably between about 15-25 nucleotides in length, e.g., about 20-24 or 21-23 nucleotides in length, more preferably about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, the strands optionally having overhanging ends comprising, for example 1, 2 or 3 overhanging nucleotides (or nucleotide analogs), which is capable of

directing or mediating RNA interference. Naturally-occurring siRNAs are generated from longer dsRNA molecules (e.g., >25 nucleotides in length) by a cell's RNAi machinery (e.g., Dicer or a homolog thereof).

The terms “protein”, “peptide”, “polypeptide”, and “amino acid sequence” are used interchangeably herein to refer to polymers of amino acid residues of any length. The polymer can be linear or branched, it may comprise modified amino acids or amino acid analogs, and it can be interrupted by chemical moieties other than amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling or bioactive component.

As used herein the term “recombinant” means a nucleotide or protein molecule that has been generated through the use of recombinant DNA technology, resulting in a nucleotide or protein molecule that does not occur in nature. One example or a recombinant nucleic acid is a nucleic acid encoding an HCMV vector that expresses a heterologous (non-CMV) antigen.

As used herein, the term “vector” encompasses any biological molecule that allows or facilitates the transfer of nucleic acid molecules from one environment to another, including a virus such as a CMV virus.

It should be understood that the proteins and the nucleic acids encoding them may differ from the exact sequences illustrated and described herein. Thus, the invention contemplates deletions, additions, truncations, and substitutions to the sequences shown, so long as the differing HCMV vectors are still capable of generating immune responses to the heterologous antigen while, a) inducing and maintaining high frequencies of extralymphoid effector memory T cell responses (so called effector memory T cells); b) reinfecting CMV-positive individuals; and c) maintaining immunogenicity while remaining spread-deficient (*i.e.*, deficient in spread from one subject or host to another subject or host).

In this regard, substitutions may be conservative in nature, *i.e.*, those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic--aspartate and glutamate; (2) basic--lysine, arginine,

histidine; (3) non-polar--alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar--glycine, asparagine, glutamine, cysteine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. It is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, or vice versa; an aspartate with a glutamate or vice versa; a threonine with a serine or vice versa; or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the sequences illustrated and described but possessing minor amino acid substitutions that do not substantially affect the activity of the vector are therefore, within the scope of the invention.

Alternatively, homologs can be expressed in terms of the percent homology relative to a described protein or nucleic acid sequence. Homologs can have at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% homology or identity to the HCMV vectors and/or heterologous antigens described herein.

Sequence identity or homology can be determined by comparing the sequences when aligned so as to maximize overlap and identity while minimizing sequence gaps. In particular, sequence identity may be determined using any of a number of mathematical algorithms. A nonlimiting example of a mathematical algorithm used for comparison of two sequences is the algorithm of Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* **87**, 2264-2268 (1990), modified as in Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* **90**, 5873-5877 (1993).

Another example of a mathematical algorithm used for comparison of sequences is the algorithm of Myers & Miller, *CABIOS* **4**, 11-17 (1988). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* **85**, 2444-2448 (1988).

Other examples of methods used to compare biological sequences, including those using the BLAST algorithms are readily available at the US National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

HCMV vectors

Disclosed herein are human cytomegalovirus (HCMV) vectors. The vectors are engineered to prevent viral spread from subject to subject (*i.e.*, cell to cellspread), yet still persistently infect subjects who have previously been infected naturally with HCMV. The vectors generate a persistent immune response to the heterologous antigen and are sensitive to the drug, ganciclovir.

In specific examples, the vectors are derived from the HCMV TR strain and have been engineered to include an active UL97 gene (not present in the original TR clinical isolate) as well as an active US2, US3, US6, and US7 gene (removed from the original TR-BAC during cloning). One example of a vector of the TR strain with these changes is referred to as TR3 herein. TR3 comprises UL97 as well as US2, US3, US6, and US7 genes from the AD169 strain. In some embodiments, the vectors derived from the HCMV TR strain further comprise an active UL131A gene. TR3 comprises an intact UL131A gene.

Additional TR3 variants have deleterious or inactivating mutations in one or more other viral genes including UL82 (which encodes the pp71 protein), UL7, UL45, UL78, and/or US13. The deleterious or inactivating mutation can be any mutation that results in a lack of function of the protein encoded by the gene, including a mutation that involves a partial or entire deletion of the coding sequence and/or the promoter of the gene. Deleterious or inactivating mutations also include point mutations and frameshift mutations of the coding sequence and/or the promoter of the gene that result in a lack of function of the protein encoded by the gene.

TR3 variants can also express heterologous antigens such as pathogen specific antigens or tumor antigens. These heterologous antigens can be expressed by any promoter including an endogenous HCMV promoter, including the UL82, UL7, UL45, UL78, and/or US13 promoters or the HCMV immediate-early promoter. In related TR3 variants, the heterologous antigen replaces the viral UL82, UL7, UL45, UL78, and/or US13 genes. In still other related TR3 variants, a first heterologous antigen replaces the UL82 gene and a second heterologous antigen replaces the viral UL7, UL45, UL78, or US13 gene.

In other examples of TR3 variants, the heterologous antigens are provided with a promoter from a CMV other than HCMV (such as MCMV-IE or RhCMV-IE), with a promoter from a herpesvirus other than CMV, from a virus other than herpesvirus, or with a non-viral promoter such as EF1α.

In some embodiments, the promoter comprises an association of DNA sequences corresponding to the minimal promoter and upstream regulatory sequences. A minimal promoter includes a CAP site plus a TATA box. These are the minimum sequences for basic, unregulated of transcription. Upstream regulatory sequences include upstream elements such as enhancer sequences. A truncated promoter is a promoter from which some portion of the full-length promoter has been removed.

Also disclosed herein are nucleic acids encoding any of the HCMV vectors described herein. While exemplary nucleic acid sequences are provided, one of skill in the art can understand that due to degeneracy in the genetic code, many different nucleic acid sequences can encode identical protein sequences. Also disclosed are cells comprising the HCMV vectors and/or nucleic acid sequences encoding the HCMV vectors. Such cells can be mammalian or human cells, such as human fetal fibroblasts and other cells. In some examples, the cells can be engineered to express an siRNA that silences the expression of a particular gene such as the DAXX gene.

Additionally disclosed herein are methods of producing an attenuated HCMV vector in a cell (e.g., an isolated cell). The methods involve infecting a cell with the attenuated HCMV vector. The cell is transfected with or expresses an siRNA that silences a gene that would otherwise prevent the attenuated HCMV vector from growing in the cell. In one example, the HCMV vector comprises a deleterious or inactivating mutation such as a deletion in pp71, and the siRNA silences expression of the DAXX gene. Also disclosed is a method of producing an attenuated HCMV vector lacking a functional pp71 protein in a cell (e.g., an isolated cell), wherein expression of the DAXX gene in the cell is downregulated at the protein or RNA level by other techniques known in the art, for example by RNA interference (e.g., microRNA targeting and short hairpin RNA (shRNA) targeting), ribozyme cleavage, regulated expression by a conditional or inducible promoter, expression of DAXX binding proteins, or targeting DAXX or DAXX protein complexes for ubiquitination and degradation.

Site-directed mutations of the type described here can be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. A suitable method is disclosed in Morinaga *et al.*, Biotechnology **2**, 646-649 (1984). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long, Analytical Biochemistry **180**, 147-151 (1989). Site directed mutagenesis methods for BACs are described in Chadburn A *et al.*, *Histopathology* **53**, 513-524 (2008); Lee E *et al.*, *Genomics* 73, 56-65 (2001); and Yu D *et al.*, *Proc Natl Acad Sci U S A* 97, 5978-5983 (2000); all of which are incorporated by reference herein.

RNA interference (RNAi) is a method of post transcriptional gene silencing induced by the direct introduction of double-stranded RNA (dsRNA) and has emerged as a useful tool to knock out expression of specific genes in a variety of organisms. RNAi is described by Fire *et al.*, *Nature* **391**, 806-811 (1998) (incorporated by reference herein). One such method involves the introduction of siRNA (small interfering RNA) into cells by transfection. Other systems, such as specific plasmid vector systems result in stable siRNA expression in a cell (for example, the pSUPER system - Brummelkamp TR *et al.*, Science **296**, 550-553 (2002); incorporated by reference herein). Methods of designing siRNAs that can efficiently silence any gene are known in the art.

Heterologous Antigens

A heterologous antigen can be derived from any protein that is not natively expressed in HCMV and includes pathogen specific antigens, tumor antigens, markers (such as fluorescent proteins or enzymes), growth factors, fusion proteins, or any other protein or fragment thereof to which an immune response can be generated (such as an MHC class I or class II restricted peptide).

The heterologous antigens in the HCMV vectors described herein can be pathogen specific antigens. For example, a protein from a viral pathogen can be used. Viral pathogens include, but are not limited to Adenovirus, coxsackievirus, hepatitis A virus, poliovirus, rhinovirus, Herpes simplex, type 1, Herpes simplex, type 2, Varicella-zoster virus, Epstein-Barr virus, Kaposi's sarcoma herpesvirus, Hepatitis B virus, Hepatitis C virus, yellow fever virus, dengue virus, West Nile virus, Human immunodeficiency virus (HIV), Influenza virus, Measles virus, Mumps virus, Parainfluenza virus, Respiratory syncytial virus, Human

metapneumovirus, Human papillomavirus, Rabies virus, Rubella virus, Human bocavirus, and Parvovirus B19. In some embodiments, the heterologous antigens in the HCMV vectors can be HIV antigens, including gag, pol, env, rev, tat, and nef. Advantageously, the HIV antigens include but are not limited to the HIV antigens discussed in U.S. Pub. Nos. 2008/0199493 A1 and 2013/0136768 A1, both of which are incorporated by reference herein.

Alternatively, the heterologous antigen can be a protein from a bacterial pathogen. Bacterial pathogens include: *Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydophila psittaci, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholera, and Yersinia pestis.*

Alternatively, the heterologous antigen can be a protein from a parasitic organism. Parasitic organisms include but are not limited to protozoans that cause diseases such as Acanthamoeba, Babesiosis, Balantidiasis, Blastocystosis, Coccidioides, Dientamoebiasis, Amoebiasis, Giardia, Isosporiasis, Leishmaniasis, Primary amoebic meningoencephalitis (PAM), Malaria, Rhinosporidiosis, Toxoplasmosis, Parasitic pneumonia, Trichomoniasis, Sleeping sickness, and Chagas disease.

Alternatively, the heterologous antigen can be a protein from a helminth organism. Helminth organisms include but are not limited to: hookworms, roundworms, tapeworms, guinea worms, liver flukes, intestinal flukes, lung flukes, *Schistosomosa*, and whipworms.

Alternatively, the heterologous antigen can be a protein derived from a tumor.

Heterologous antigens can be codon optimized. Many viruses, including HIV and other lentiviruses, use a large number of rare codons and, by altering these codons to

correspond to codons commonly used in the desired subject (for example, humans), enhanced expression of the antigens can be achieved. For example, rare codons used in HIV proteins can be mutated into those that appear frequently in highly expressed human genes (Andre *et al*., J Virol 72, 1497-1503, (1998). Additionally antigens can be consensus sequences or mosaic antigens containing sequence fragments from different strains of pathogens.

Immunogenic compositions:

Disclosed herein are immunogenic compositions containing the disclosed recombinant HCMV vectors, and a pharmaceutically acceptable carrier or diluent. An immunogenic composition containing the recombinant HCMV vector elicits an immunological response. The response can, but need not be, protective. A vaccine composition elicits protective response, generally involving the development of immunological memory.

Methods of inducing an immunological response in a subject are also disclosed. Such methods involve administering to the subject an immunogenic or vaccine composition comprising the disclosed recombinant HCMV vectors and a pharmaceutically acceptable carrier or diluent. For purposes of this specification, the term "subject" includes all animals and humans.

The immunogenic or vaccine compositions can be administered via a parenteral route (intradermal, intramuscular, or subcutaneous). Other administration can be via a mucosal route, *e.g.*, oral, nasal, genital, etc.

The immunogenic or vaccine compositions can be formulated and administered in accordance with standard techniques well known to those skilled in the pharmaceutical arts. The compositions can be administered alone, or can be co-administered or sequentially administered with other HCMV vectors or with other immunogenic, vaccine, or therapeutic compositions.

Examples of such compositions include liquid preparations such as preparations for injectable administration – for example, parenteral, subcutaneous, intradermal, intramuscular or intravenous administration - such as sterile suspensions or emulsions. In such compositions the HCMV vector is in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like.

Immunogenic or vaccine compositions can contain an adjuvant. Alum (aluminum phosphate or aluminum hydroxide) is a typical adjuvant. Saponin and its purified component Quil A, Freund's complete adjuvant, Freund's incomplete adjuvant and other adjuvants are often used in research and veterinary applications.

The composition can be packaged in a single dosage form for injectable administration or other administration with the effective dosage and route of administration determined by the nature of the composition, by the nature of the expression product and other factors. The dosage of the disclosed HCMV vectors can be expressed in plaque forming units (pfu) including a dosage of more than 10^2 pfu, more than 10^3 pfu, more than 10^4 pfu, more than 10^5 pfu, more than 10^6 pfu, or more than 10^7 pfu.

EXAMPLES

The following examples are illustrative of disclosed methods. In light of this disclosure, those of skill in the art will recognize that variations of these examples and other examples of the disclosed method would be possible without undue experimentation.

Example 1 – The HCMV-TR3 vector platform

Clinical use of effector memory T cell-inducing CMV vectors requires vectors that are genetically stable and maintain a persistent infection, but lack the ability to spread to immunocompromised subjects in which HCMV can be pathogenic. Previous attenuation strategies for HCMV variants that entered clinical trials relied on serial passaging of virus in fibroblasts (Plotkin SA *et al.*, J Infect Dis **134**, 470-475 (1976); incorporated by reference herein), recombination of attenuated with non-attenuated HCMV strains (Heineman J *et al.* 2006 *supra*) or generation of replication-deficient recombinant vectors (WO2013/036465; incorporated by reference herein). However, the resulting viruses either retained pathogenicity or lost beneficial features such as the ability to establish latent infections or secondary infections in subjects previously infected naturally with CMV.

Disclosed herein is an HCMV vector platform – HCMV-TR3 - that overcomes these limitations. HCMV TR3 is a modified version of the molecular clone HCMV-TR (Murphy E *et al.*, *Proc Natl Acad Sci U S A* **100** 14976-14981 (2003); incorporated by reference herein). HCMV TR is superior to other HCMV strains in establishing latency and persistence in vivo. HCMV-TR is also superior to other clinical isolates of HCMV in vitro since it does not display the HCMV-typical fibroblast-adaptations upon multiple passages. TR3 was altered in order

to make it ganciclovir-sensitive, to make it able to reinfect previously infected subjects, and to facilitate the recovery of CMV vector from the bacterial artificial chromosome (BAC) system.

Specifically, deletion of the UL82 gene (which encodes the pp71 protein) from TR3 results in the generation of a spread-deficient (*i.e.*, defective in cell to cell spread) vector. However, previously viruses that lack pp71 expression were shown to require complementation for growth in vitro (Bresnahan, W. A., and T. E. Shenk. *Proc Natl Acad Sci* U S A 97:14506-11 (2000); incorporated by reference herein). UL82 virion protein activates expression of immediate early viral genes in human cytomegalovirus-infected cells, which in turn results in the risk that the virus will revert to a wild type with active pp71. As a result, a new method of growing HCMV vectors lacking pp71 was developed and described in detail below.

A non-human primate model further demonstrates that pp71-deleted HCMV-TR3 maintains the ability to induce and maintain effector memory T cell responses while tropism-deficient versions of HCMV-TR3 that recapitulate viral adaptations that commonly result from passage through fibroblasts do not.

Additionally, pp71-deleted HCMV-TR3 vectors maintain latent infections but lack the ability to reactivate in humanized mice.

Further, internal expression sites that can be used to insert and express heterologous antigens are disclosed. These can be used to produce HCMV vectors that include multiple heterologous antigens.

Example 2 - HCMV-TR is superior to other HCMV strains in establishment of latent infection

A humanized mouse model that permits studying HCMV latency and reactivation is described in Smith MS *et al.*, *Cell Host Microbe* **8**, 284-291 (2010) (incorporated by reference herein). This model was used to demonstrate that HCMV-TR is superior to other HCMV strains (AD169, Toledo) in establishing persistent infection. Persistent infection is important for the induction of effector memory T cells. The ability to generate a persistent infection is independent of the UL128-150 region, which is mutated in many HCMV strains including all strains previously used in clinical trials of HCMV vaccine (AD169, Towne and Toledo). The repair of UL131A in the AD169 strain does not restore the ability to establish latency, but

the HCMV-TRΔ4 strain that lacks UL128-150 maintains the ability to establish latency (**Figure 1B**). Note that these previous clinical trials did not involve HCMV comprising heterologous antigens. Genetic maps of these strains are shown in **Figure 1A**.

Example 3 - HCMV-TR3 is sensitive to ganciclovir and includes the US2-7 region whereas the original HCMV-TR does not

HCMV TR was cloned by BAC recombineering from a viral isolate that is resistant to the antiviral drug ganciclovir (Smith IL *et al.*, *J Infect Dis* **176**, 69-77 (1997); incorporated by reference herein). ganciclovir resistance is not a desirable trait in a HCMV vector because treatment with ganciclovir would be important in the event of CMV-associated disease caused by HCMV-based vectors. Confirmation of ganciclovir resistance is shown in **Figure 3**.

An intact UL97 gene was inserted into HCMV TR (**Figure 2**) to generate a ganciclovir-sensitive vector. The molecular clone of HCMV-TR was further modified. Insertion of a BAC cassette during the original cloning of HCMV TR resulted in a deletion of the US2-7 region (Murphy *et al*. 2003 *supra*). US2-7 was later determined to be a region that is essential for the reinfection of CMV-positive individuals (Hansen SG *et al.*, *Science* **328**, 102-106 (2010); incorporated by reference herein. A modified version of HCMV-TR was generated in which the US2-7 region of HCMV strain AD169 was inserted to modify the BAC cassette. This modification was made because in the original HCMV TR clone that BAC cassette could not be removed when virus is reconstituted by transfection of fibroblasts (Lauron E *et al.*, *J Virol* **88**, 403-416 (2014); incorporated by reference herein). HCMV-TR3, therefore also includes the US2-7 region of AD169 and a loxP site between US7 and US8 upon viral reconstitution as shown by full genome sequencing (**Figure 2**).

Example 4 - HCMV-TR3 displays superior genome stability upon multiple passages through fibroblasts

Passaging of HCMV in fibroblasts results in the preferential selection of vectors with deleterious (*i.e.*, inactivating) mutations in the UL128-131A region (Dargan DJ *et al.*, *J Gen Virol* **91**, 1535-1546 (2010); incorporated by reference herein) and the RL13 gene (Stanton RJ *et al*. *J Clin Invest* **120**,3191-208; (2010); incorporated by reference herein). However, passaging through fibroblasts results in the highest viral yields when producing vaccine. **Figure 4A** shows that, surprisingly, the genome of HCMV-TR3 remains stable even after 20 passages in fibroblasts.

Example 5 - The presence of UL128-131A does not reduce the yield of cell free HCMV-TR3 unlike other strains of HCMV

For vaccine manufacturing, cell supernatants, rather than cell pellets, are preferred to isolate vaccine vectors. In most HCMV strains, the yield of cell free virus from fibroblasts is drastically reduced when the genes UL131A, UL130 and UL128 are intact (Wang D and Shenk T, *J Virol* **79**, 10330-10338 (2005); incorporated by reference herein). Surprisingly, removal of UL131A-128 does not affect the ratio of cell-free versus cell associated virus for HCMV-TR3 (**Figure 5**).

Example 6 - HCMV-TR3 induces effector memory T cells in monkeys whereas HCMV mutants lacking the UL128-131 region are unable to do so.

HCMV-TR3 expressing the Gag-antigen of SIV is capable of inducing an effector memory T cell response against Gag in non-human primates (NHP; **Figure 6A**). Importantly, this effector memory T cell response is maintained over time (**Figure 11**). In contrast, HCMV-TR3 lacking the genes UL128-131, a gene region that is frequently mutated in HCMV strains attenuated by serial passaging in vitro, is unable to do so (**Figure 6B**). This is also the first known demonstration of an HCMV vector inducing an immune response to a heterologous antigen in a non-human primate model. Further deletions in this genomic region demonstrated that viruses that lack UL128 and UL130 are able to elicit immune responses to heterologous antigens in vivo similar to the parental vectors (**Figure 12**). Therefore, we conclude that UL131A is essential for infection by HCMV.

Example 7 - Generation of uncomplemented pp71-deleted HCMV-TR3 using DAXX siRNA. A method to grow attenuated virus without complementation or FKBP-fusion

A major limitation for the manufacturing of HCMV lacking essential genes, or genes that are required for optimal replication in vitro, is the need for complementation – that is, the exogenous expression of the deleted gene in a producer cell line. Producer cell lines are well known to be difficult to make and maintain, particularly in the context of GMP vaccine manufacturing.

One approach used in complementation is to fuse the essential gene to a degradation domain (such as FKBP), a strategy described in WO2013/036465 (incorporated by reference herein). While FKBP-fusions might be useful for the manufacturing of non-persistent vaccines that are replication deficient in vivo, in the case of the mutant HCMV

described herein there is a risk that the degradation domain will be mutated and the attenuation will thus be lost, rendering the HCMV able to spread from host to host.

Disclosed herein is an approach involving silencing an antiviral host cell factor using, for example, siRNA. The result is a cell line that does not require complementation because the mutant HCMV can be grown in vitro, even though it remains attenuated in vivo. An example of this process is illustrated in **Figure 7A**. As described above, HCMV-TR3 lacking the UL82 gene that encodes phosphoprotein 71 (pp71) is unable to grow in fibroblasts. However, when expression of the antiviral protein DAXX is silenced by siRNA expressed in fibroblasts, HCMV-TR3ΔUL82 can be grown at high yield (**Figure 7B** and **Figure 13**).

Example 8 - HCMV-TR3 lacking UL82(pp71) maintains persistence in vivo but is deficient in its ability to reactivate from latency

Human cytomegalovirus (HCMV) establishes latent infection in host cells that is regulated via temporal expression viral genes. HCMV pp71 is a tegument protein that counteracts the host intrinsic immunity degradation of the cellular protein Daxx (death domain associated protein) (Penkert, RR, and RF Kalejta, *Future Virol* **7**, 855-869 (2012); incorporated by reference herein). Degradation of Daxx by pp71 is necessary for optimal immediate early gene expression and lytic replication. *In vitro* data suggests that HCMV prevents pp71-mediated degradation of Daxx during establishment of latency by sequestering pp71 in the cytoplasm of infected cells. However, the *in vivo* role of pp71 in HCMV persistence, maintenance of latency and reactivation remains unknown. We have previously shown that HCMV infection of human hematopoietic stem cells (HSCs) engrafted in immune deficient mice (HU-NSG) results in viral latency that can be reactivated following G-CSF treatment. While this model is important, HU NSG mice lack mature human T-cells. In contrast NSG mice transplanted with HSCs in conjunction with human fetal liver and thymus (BLT mice) develop all the human hematopoietic cell lineages necessary for a functional human immune system, including mature CD4 and CD8 T-cells. In this new humanized mouse model it is demonstrated that HCMV establishes latency and reactivation similar to HU-NSG mice. Latently infected mice also generate human IgG as well as HCMV-specific T-cell responses. Importantly, infection of BLT mice with a conditionally expressing pp71 (TR UL82-FKBP) or a pp71 knockout (TR(delta)UL82) resulted in the establishment of infection but failed to reactivate. These data indicate that pp71 plays an important role in HCMV

reactivation and that replication deficient virus can generate a T-cell response. The ability to replicate in vitro is not a good predictor of whether a virus can establish latency, as shown in Fig. 1B. For example, AD169 replicates well in vitro, but cannot establish latency, as shown in Fig. 1B. However, HCMV-TR3ΔUL82 grown on DAXX siRNA expressing MRC-5 cells establishes latency in humanized mice, but does not reactivate or disseminate (**Figure 8**). Similar results were obtained in NSG mice for HCMV-TR3ΔUL82 and HCMV-TR3ΔUL82ΔUL128-130 (**Figure 14**).

Example 9 - pp71-deleted HCMV-TR3 expressing HIVgag maintains the ability to induce HIVgag specific effector memory T cells in in vivo

Due to its large genome, HCMV offers the opportunity to insert multiple heterologous antigens into a viral vector. The expression of multiple heterologous antigens by HCMV requires the identification of endogenous genes that can be used to insert foreign sequences without affecting vector function. Previously, transposon analysis identified all nonessential genes in the HCMV genome *in vitro* (Yu D *et al.*, *Proc Natl Acad Sci U S A* **100**, 12396-12401 (2003); incorporated by reference herein.

However, this does not provide a prediction as to which non-essential genes in vitro would be non-essential in vivo and, further, whether or not the replacement of a viral gene with a gene encoding a heterologous antigen would induce an immune response when the expression of the heterologous antigen is driven by the promoter of the replaced gene. **Figure 9** and **Figure 15** show that replacement of UL82(pp71) with HIVgag elicits and maintains an effector memory type T cell immune response in vivo.

Additional sites for replacement with a heterologous antigen include HCMV UL7, UL78 and US13. When each of these is replaced with a heterologous antigen (SIVpol) in vectors that already carry a replacement of the pp71-ORF with antigen (SIVenv), immune responses were generated each time. The results are summarized in **Figure 10A**. **Figure 10B** shows that replacement of UL7, UL45 and UL78 with HIVgag in HCMV results in HIVgag expression in vitro.

Example 10 – Stability of pp71 deleted HCMV-TR3 through growth and production under conditional complementation.

Previous work demonstrated that clinical isolates of HCMV undergo rapid adaptation in vitro when grown in fibroblasts. In particular, generation of frameshift mutations leading

to premature stop codons in RL13 and loss of expression of one or more of the pentameric complex proteins (UL128, UL130 and UL131A) can occur after even a low number of passages in tissue culture (Stanton RJ *et al*. *J Clin Invest* **120**(9), 3191-3208 (2010); incorporated by reference herein). Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication (*Id*.). As a consequence, all HCMV strains previously used in clinical studies (AD169, Towne, Toledo) display multiple rearrangements and deletions (Murphy, ED *et al*. *Proc Natl Acad Sci U.S.A.* **100**(25), 14976-14981 (2003); incorporated by reference herein). These fibroblast-adaptations might result in the deletion of UL131A, as observed in AD169, thus rendering the virus non-infectious in vivo. To determine whether UL82-deleted HCMV-TR3/HIVgag grown in fibroblast cells treated with DAXX siRNA would similarly display instability upon multiple passages, we analyzed the viral genome by next generation sequencing (NGS).

Specifically, the recombinant bacterial artificial chromosome DNA was sequenced prior to introduction into fibroblasts, and, upon reconstitution in fibroblasts, viral DNA was isolated at passage 5 and passage 9. Genomic DNA was isolated from the supernatant of infected human fibroblasts by Hirt extraction (Hirt B. *J Mol Biol*. **26**(2):365-369 (1967); incorporated by reference herein) after virus purification through a 20% sucrose cushion. DNA libraries were generated using the TruSeq DNA Sample Preparation kit and adapters with known primer binding sites were ligated to each end of the DNA fragments. Paired end sequencing, analyzing 150 bp on each end of the unknown DNA, was performed on an Illumina MiSeq NGS sequencer using the MiSeq Reagent Kits v2 for 300 cycles. The resulting sequence reads were imported into Geneious 8.1.4 and trimmed with the lowest possible error probability limit of 0.001, meaning that every base pair with a higher error probability of 0.1% is deleted. De novo sequence assembly was performed with 250.000 to 1.000.000 reads to determine the DNA sequence in an unbiased fashion. No major insertions, deletions or genomic rearrangements were observed compared to the predicted sequences. Next, a reference-guided assembly of all reads was performed using the *de novo* sequence as the reference to determine the full and correct majority sequence. The mean minimum coverage was > 150 fold.

Figure 16 shows an alignment of the resulting sequences. Open reading frames (ORFs) encoded in the self-excising BAC cassette are depicted with white arrows, and viral

ORFs are depicted with grey arrows. Yellow arrows depict the HIVgag ORF replacing the UL82 ORF. Grey ovals depict internal and terminal repeats. Non-coding regions are shown as interruptions of the coding regions shown as black bars. As expected, the BAC cassette was excised upon viral reconstitution in tissue culture. However, all other nucleotides in the majority sequence were identical to the predicted sequence (consensus). Importantly, no changes of any amino acids were observed in the ORFs even through nine passages. This includes ORFs encoding the UL128-131A genes, RL13 as well as the AD169-derived genes UL97 and US2-7. These observations suggest a surprising stability of UL82-deleted HCMV-TR3 despite multiple passages in fibroblasts in the presence of DAXX siRNA.

Importantly, there were no changes in the ORF encoding HIVgag expressed by the UL82 promoter. This was independently confirmed by immunoblot and Sanger-sequencing of the HIVgag insert at passages 5, 6 and 7 after reconstitution of UL82(pp71)-deleted HCMV-TR3. **Figure 17A** shows an immunoblot of lysates from fibroblasts infected with the indicated viruses. Lysates were separated by SDS-PAGE, transferred onto nylon membranes and reacted with antibodies specific for pp71, HIVgag (p24) and the viral protein pp28 and the cellular protein actin. As expected, pp71 was present in the parental TR3 virus, but not in HIVgag-expressing vectors due to replacement of UL82 with HIVgag. Importantly, HIVgag was stably expressed upon each passage. **Figure 17B** shows an alignment based on sequences analysis of PCR-fragments spanning the HIVgag gene and obtained from viral DNA at the indicated passage. No nucleotide changes were observed.

In contrast to the surprisingly stable expression of HIVgag expressed by the endogenous UL82 promoter, expression of heterologous antigens by heterologous promoters are routinely unstable upon multiple passages. For example, SIVgag expressed by the heterologous EF1α promoter in the RhCMV 68-1.2 vector displayed a premature disruption of the coding region due to a point mutation. **Figure 18** shows the frequency of single nucleotide polymorphisms (SNPs) compared to the reference sequence from a next generation sequencing analysis of a UL36-deleted RhCMV vector derived from a clone of RhCMV 68-1.2 that expresses SIVgag using the EF1α promoter. Approximately 38% of the genomes demonstrate a premature stop codon in the SIVgag sequence.

CLAIMS

1. A recombinant ganciclovir sensitive TR strain human cytomegalovirus (HCMV) vector comprising:
   (1) a nucleic acid encoding at least one heterologous antigen;
   (2) an inactivating mutation in the UL78 gene; and
   (3) active US2, US3, US6, US7, UL97, and UL131A genes.

2. The recombinant HCMV vector of claim 1, wherein the active US2, US3, US6, and US7 genes are derived from the AD169 strain of HCMV.

3. The recombinant HCMV vector of claim 1 or 2, wherein the expression of the nucleic acid encoding at least one heterologous antigen is driven by the UL82 promoter, the UL7 promoter, the UL45 promoter, the UL78 promoter, or the US13 promoter.

4. The recombinant HCMV vector of any one of claims 1-3, wherein the inactivating mutation in the UL78 gene is a deletion of all or part of the UL78 gene.

5. The recombinant HCMV vector of claim 4, wherein the nucleic acid encoding at least one heterologous antigen replaces all or part of the UL78 gene.

6. The recombinant HCMV vector of claim 5, wherein the expression of the nucleic acid encoding at least one heterologous antigen replacing all or part of the UL78 gene is driven by the UL78 promoter.

7. The recombinant HCMV vector of any one of claims 1-6, further comprising an inactivating mutation in a HCMV gene selected from the group consisting of: UL7, UL38, UL45, UL82, and US13.

8. The recombinant HCMV vector of claim 7, wherein the inactivating mutation in the UL7 gene is a deletion of all or part of the UL7 gene.

9. The recombinant HCMV vector of claim 8, wherein the nucleic acid encoding at least one heterologous antigen replaces all or part of the UL7 gene.

10. The recombinant HCMV vector of claim 9, wherein the expression of the nucleic acid encoding at least one heterologous antigen replacing all or part of the UL7 gene is driven by the UL7 promoter.

11. The recombinant HCMV vector of claim 7, wherein the inactivating mutation in the UL38 gene is a deletion of all or part of the UL38 gene.

12. The recombinant HCMV vector of claim 11, wherein the nucleic acid encoding at least one heterologous antigen replaces all or part of the UL38 gene.

13. The recombinant HCMV vector of claim 12, wherein the expression of the nucleic acid encoding at least one heterologous antigen replacing all or part of the UL38 gene is driven by the UL38 promoter.

14. The recombinant HCMV vector of claim 7, wherein the inactivating mutation in the UL45 gene is a deletion of all or part of the UL45 gene.

15. The recombinant HCMV vector of claim 14, wherein the nucleic acid encoding at least one heterologous antigen replaces all or part of the UL45 gene.

16. The recombinant HCMV vector of claim 15, wherein the expression of the nucleic acid encoding at least one heterologous antigen replacing all or part of the UL45 gene is driven by the UL45 promoter.

17. The recombinant HCMV vector of claim 7, wherein the inactivating mutation in the US13 gene is a deletion of all or part of the US13 gene.

18. The recombinant HCMV vector of claim 17, wherein the nucleic acid encoding at least one heterologous antigen replaces all or part of the US13 gene.

19. The recombinant HCMV vector of claim 18, wherein the expression of the nucleic acid encoding at least one heterologous antigen replacing all or part of the US13 gene is driven by the US13 promoter.

20. The recombinant HCMV vector of any one of claims 1-19, further comprising an inactivating mutation in the UL128 gene or the UL130 gene.

21. The recombinant HCMV vector of claim 20, wherein the recombinant HCMV comprises an inactivating mutation in the UL128 gene and the UL130 gene.

22. The recombinant HCMV vector of any one of claims 1-21, wherein the at least one heterologous antigen is a pathogen specific antigen or tumor antigen.

23. The recombinant HCMV vector of any one of claims 1-22, wherein the expression of the nucleic acid encoding at least one heterologous antigen is driven by an endogenous promoter and wherein the nucleic acid encoding at least one heterologous antigen is stable upon multiple passages through fibroblasts.

24. The recombinant HCMV vector of any one of claims 1-22, wherein the nucleic acid encoding at least one heterologous antigen is a nucleic acid encoding a first heterologous antigen, and wherein the recombinant HCMV vector further comprises a nucleic acid encoding a second heterologous antigen.

25. The recombinant HCMV vector of claim 24, wherein the nucleic acid encoding the first heterologous antigen replaces all or part of the UL78 gene, and wherein the nucleic acid encoding the second heterologous antigen replaces all or part of an HCMV gene selected from the group consisting of: UL7, UL45, and US13.

26. The recombinant HCMV vector of claim 25, wherein the expression of the nucleic acid encoding the first heterologous antigen is driven by the UL78 promoter, and wherein the expression of the nucleic acid encoding the second heterologous antigen is driven by the UL7 promoter, the UL45 promoter, or the US13 promoter.

27. The recombinant HCMV vector of any one of claims 24-26, wherein the first heterologous antigen is a pathogen specific antigen or tumor antigen.

28. The recombinant HCMV vector of claim 27, wherein the second heterologous antigen is a pathogen specific or tumor antigen that is different from the first heterologous antigen.

29. The recombinant HCMV vector of any one of claims 24-28, wherein the expression of the nucleic acids encoding the first and second heterologous antigens is driven by endogenous promoters and wherein the nucleic acids encoding the first and second heterologous antigens are stable upon multiple passages through fibroblasts.

30. An immunogenic composition comprising the recombinant HCMV vector of any one of claims 1-23 and a pharmaceutically acceptable carrier.

31. Use of the immunogenic composition of claim 30 in the manufacture of a medicament for inducing an immune response in a subject.

32. The use of claim 31, wherein the recombinant HCMV vector induces and maintains a long-term effector memory T cell response to the at least one heterologous antigen in the subject.

33. The immunogenic composition of claim 30 for use in inducing an immune response in a subject.

34. The immunogenic composition of claim 33, wherein the recombinant HCMV vector induces and maintains a long-term effector memory T cell response to the at least one heterologous antigen in the subject.

35. An immunogenic composition comprising the recombinant HCMV vector of any one of claims 24-29 and a pharmaceutically acceptable carrier.

36. Use of the immunogenic composition of claim 35 in the manufacture of a medicament for inducing an immune response in a subject.

37. The use of claim 36, wherein the recombinant HCMV vector induces and maintains a long-term effector memory T cell response to the first and second heterologous antigens in the subject.

38. The immunogenic composition of claim 35 for use in inducing an immune response in a subject.

39. The immunogenic composition of claim 38, wherein the recombinant HCMV vector induces and maintains a long-term effector memory T cell response to the first and second heterologous antigens in the subject.

40. An isolated polynucleotide that encodes the recombinant HCMV vector of any one of claims 1-29.