# REPLICATION-DEFECTIVE FLAVIVIRUS VACCINES AND VACCINE VECTORS

## Field of the Invention

This invention relates to replication-defective flavivirus vaccines and vaccine vectors, and corresponding compositions and methods.

## Background of the Invention

Flaviviruses are distributed worldwide and represent a global public health problem. Flaviviruses also have a significant impact as veterinary pathogens. Flavivirus pathogens include yellow fever (YF), dengue types 1-4 (DEN1-4), Japanese encephalitis (JE), West Nile (WN), tick-borne encephalitis (TBE), and other viruses from the TBE serocomplex, such as Kyasanur Forest disease (KFD) and Omsk hemorrhagic fever (OHF) viruses. Vaccines against YF [live attenuated vaccine (LAV) strain 17D], JE [inactivated vaccines (INV) and LAV], and TBE (INV) are available. No licensed human vaccines are currently available against DEN and WN. Veterinary vaccines have been in use including, for example, vaccines against WN in horses (INV, recombinant and live chimeric vaccines), JE (INV and LAV) to prevent encephalitis in horses and stillbirth in pigs in Asia, louping ill flavivirus (INV) to prevent neurologic disease in sheep in the UK, and TBE (INV) used in farm animals in Czech Republic (INV) (Monath and Heinz, Flaviviruses, in Fields et al. Eds., Fields Virology, 3rd Edition, Philadelphia, New York, Lippincott-Raven Publishers, 1996, pp. 961-1034).

Tick-borne encephalitis (TBE) is the most important tick-borne viral disease of humans. It is endemic in parts of Europe and Northern Asia, causing more than 10,000 hospitalizations annually, with a case-fatality rate 0.5–1.5% in Europe and 6–40% in Siberia and the Far East. A significant proportion of patients suffer from long-lasting neuropsychiatric sequelae. Inactivated vaccines produced in chick embryo cell cultures have proven effective in preventing the disease. For example, an 86% vaccination coverage of the Austrian population (the highest among European countries) has resulted

Suzuki et al., J. Virol. 83:1870-1880, 2009; Ishikawa et al., Vaccine 26:2772-2781, 2008; Widman et al., Vaccine 26:2762-2771, 2008; WO 2007/098267; WO 2008/137163).

In the second variation, a two-component PIV (d-PIV) is constructed (Fig. 2). Substrate cells are transfected with two defective viral RNAs, one with a deletion in the C gene and another lacking the prM-E envelope protein genes. The two defective genomes complement each other, resulting in accumulation of two types of PIVs in the cell culture medium (Shustov et al., J. Virol. 21:11737-11748, 2007; Suzuki et al., J. Virol. 82:6942-6951, 2008). Optionally, the two PIVs can be manufactured separately in appropriate helper cell lines and then mixed in a two-component formulation. The latter may offer an advantage of adjusting relative concentrations of the two components, increasing immunogenicity and efficacy. This type of PIV vaccine should be able to undergo a limited spread in vivo due to coinfection of some cells at the site of inoculation with both components. The spread is expected to be self-limiting as there are more cells in tissues than viral particles produced by initially coinfected cells. In addition, a relatively high MOI is necessary for efficient co-infection, and cells outside of the inoculation site are not expected to be efficiently coinfected (e.g., in draining lymph nodes). Cells infected with the ΔC PIV alone produce the highly immunogenic VLPs. Coinfected cells produce the two types of packaged defective viral particles, which also stimulate neutralizing antibodies. The limited infection is expected to result in a stronger neutralizing antibody response and T-cell response compared to s-PIVs. To decrease chances of recombination during manufacture or in vivo, including with circulating flaviviruses, viral sequences can be modified in both s-PIVs and d-PIVs using, e.g., synonymous codon replacements, to reduce nucleotide sequence homologies, and mutating the complementary cyclization 5' and 3' elements.

## Summary of the Invention

In one aspect, there is provided a replication-deficient pseudoinfectious flavivirus comprising a yellow fever or West Nile virus genome comprising (i) a deletion in the nucleotide sequence encoding amino acids 26-93 or 31-100 or 31-93 or 26-100 of capsid (C) protein, and one or more deletions within nucleotide sequences encoding a pre-membrane (prM) and/or envelope (E) protein, and (ii) a sequence encoding one or more heterologous pathogen immunogens inserted in the place of or in combination with the one or more deletions of the pre-membrane (prM) and/or envelope (E) protein, wherein said pathogen is selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, an influenza virus, a human immunodeficiency virus, a simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, a malaria parasite, and *Mycobacterium tuberculosis*.

The disclosure~~invention~~ provides replication-deficient or defective pseudoinfectious flaviviruses including a flavivirus genome that includes (i) one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1

the heterologous immunogens can be inserted in the place of, or in combination with, the deletion(s) or mutation(s) of the one or more proteins.

The sequences encoding the heterologous immunogens can be inserted in the flavivirus genomes within sequences encoding the envelope (E) protein, within sequences encoding the non-structural 1 (NS1) protein, within sequences encoding the pre-membrane (prM) protein, intergenically between sequences encoding the envelope (E) protein and non-structural protein 1 (NS1), intergenically between non-structural protein 2B (NS2B) and non-structural protein 3 (NS3), and/or as a bicistronic insertion in the 3' untranslated region of the flavivirus genome.

In another aspect there is provided a pharmaceutical composition comprising the replication-deficient pseudoinfectious flavivirus of the present invention and a pharmaceutically acceptable carrier or diluent.

The disclosure~~invention~~ also includes compositions including a first replication-deficient pseudoinfectious flavivirus, as described above, and a second (or further), different replication-deficient pseudoinfectious flavivirus including a genome that includes one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5). In these compositions, the one or more proteins encoded by the sequences in which the deletion(s) or mutation(s) occur in the second, different replication-deficient pseudoinfectious flavivirus are different from the one or more proteins encoded by the sequences in which the deletion(s) occur in the first replication-deficient pseudoinfectious flavivirus.

In a further aspect, there is provided the pharmaceutical composition of the present invention for use in inducing an immune response against the heterologous pathogen immunogen in a subject.

In another aspect, there is provided the pharmaceutical composition of the present invention for use in inducing an immune response against the heterologous pathogen immunogen in a subject wherein the subject is at risk of but does not have an infection by said pathogen.

In yet another aspect, there is provided the pharmaceutical composition of the present invention for use in inducing an immune response against the heterologous pathogen immunogen in a subject wherein the subject has an infection by said pathogen.

The disclosure~~invention~~ further includes methods of inducing immune responses to an immunogen in a subject, which involves administering to the subject one or more replication-deficient pseudoinfectious flavivirus and/or composition as described herein to the subject. In various examples, the subject is at risk of but does not have an infection by the pathogen or a disease or condition associated with the cancer or allergy-related immunogen. In other examples, the subject has an infection by the pathogen or a disease or condition associated with the cancer or allergy-related immunogen. The disclosure~~invention~~ thus includes prophylactic and therapeutic methods. In these methods, the immunogen can be from, for example, a pathogen selected from the group consisting of a rabies virus, *Borrelia burgdorferi*, a tick, an influenza virus, a human immunodeficiency virus, a

simian immunodeficiency virus, a human papilloma virus, a respiratory syncytial virus, malaria parasite, and *Mycobacterium tuberculosis* (also see below). Further, the methods can be for inducing an immune response against a protein encoded by the flavivirus genome, in addition to the source of the immunogen. In various examples, the subject is at risk of but does not have an infection by the flavivirus corresponding to the genome of the pseudoinfectious flavivirus, which includes sequences encoding a flavivirus pre-membrane and/or envelope protein. In other examples, the subject has an infection by the flavivirus corresponding to the genome of the pseudoinfectious flavivirus, which includes sequences encoding a flavivirus pre-membrane and/or envelope protein.

The disclosure~~invention~~ also includes live, attenuated chimeric flaviviruses including a yellow fever virus in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope proteins of a tick-borne encephalitis virus or a Langat virus, and the signal sequence between the capsid and pre-membrane proteins of the chimeric flavivirus includes a hybrid of yellow fever virus and tick-borne encephalitis or Langat virus capsid/pre-membrane signal sequences, or a variant thereof. In various examples, the capsid/pre-membrane signal sequence of the chimeric flavivirus includes yellow fever virus sequences in the amino terminal region and tick-borne encephalitis or Langat virus sequences in the carboxy terminal region (see below).

Further, the disclosure~~invention~~ includes live, attenuated chimeric flaviviruses including a West Nile virus in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope proteins of a tick-borne encephalitis or a Langat virus, and the signal sequence between the capsid and pre-membrane proteins of the chimeric flavivirus includes a tick-borne encephalitis or a Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

The disclosure~~invention~~ also includes pharmaceutical compositions including one or more pseudoinfectious flavivirus, composition, or live, attenuated flavivirus as described herein, and a pharmaceutically acceptable carrier or diluent. Further, the compositions can include an adjuvant.

Also included in the disclosure~~invention~~ are replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletion(s) or mutation(s) in nucleotide sequences encoding non-structural protein 1 (NS1), non-structural protein 3 (NS3), or non-structural protein 5 (NS5).

In one aspect, there is provided a nucleic acid molecule corresponding to the genome of a pseudoinfectious flavivirus of the present invention.

Further, the disclosure~~invention~~ includes nucleic acid molecules corresponding to the genome of a pseudoinfectious flavivirus, or the genome the live, attenuated flavivirus, as described herein, and complements thereof.

In one aspect, there is provided a method of making a replication-deficient pseudoinfectious flavivirus of the present invention, the method comprising introducing a nucleic acid molecule of the present invention into a cell that expresses the protein corresponding to any sequences deleted from the flavivirus genome of the replication-deficient pseudoinfectious flavivirus.

The disclosure~~invention~~ also provides methods of making replication-deficient pseudoinfectious flaviviruses as described herein, involving introducing one or more nucleic acid molecules, as described above, into a cell that expresses the protein(s) corresponding to any sequences deleted from the flavivirus genome of the replication-deficient pseudoinfectious flaviviruses. In these methods, the protein can be expressed in the cell from the genome of a second (or further), different, replication-deficient pseudoinfectious flavivirus. In other examples, the protein is expressed from a replicon (e.g., an alphavirus replicon, such as a Venezuelan Equine Encephalitis virus replicon; see below).

The disclosure~~invention~~ also includes compositions containing two or more replication-deficient pseudoinfectious flaviviruses, in which two of the replication-deficient pseudoinfectious flaviviruses are selected from the groups consisting of: (a) a replication-deficient pseudoinfectious flavivirus including a genome containing Japanese encephalitis virus sequences, and a replication-deficient pseudoinfectious flavivirus including a genome containing dengue virus sequences; (b) a replication-deficient pseudoinfectious flavivirus including a genome containing yellow fever virus sequences, and a replication-deficient pseudoinfectious flavivirus including a genome containing dengue virus sequences; and (c) a replication-deficient pseudoinfectious flavivirus including a genome containing tick-borne encephalitis or Langat virus sequences and an inserted sequence encoding a *Borrelia burgdorferi* immunogen, and a replication-deficient pseudoinfectious flavivirus including a genome containing tick-borne encephalitis or Langat virus sequences and an inserted sequence encoding a tick saliva protein immunogen, or a replication-deficient pseudoinfectious flavivirus including a genome containing

tick-borne encephalitis or Langat virus sequences and inserted sequences encoding a *Borrelia burgdorferi* immunogen and a tick saliva protein immunogen.

Pharmaceutical compositions including the live, attenuated chimeric flaviviruses described herein are also included in the disclosure~~invention~~. Further, the disclosure~~invention~~ includes methods of inducing an immune response to tick-borne encephalitis virus or Langat virus in a subject, involving administering to the subject such a pharmaceutical composition. In various examples, the subject does not have but is at risk of developing infection by tick-borne encephalitis virus or Langat virus. In other examples, the subject is infected with tick-borne encephalitis virus or Langat virus.

The disclosure~~invention~~ further includes replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), wherein the flavivirus genome includes yellow fever virus sequences in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope proteins of a tick-borne encephalitis virus or a Langat virus, and sequences encoding the signal sequence between the capsid and pre-membrane proteins of the flavivirus genome include a hybrid of sequences encoding yellow fever virus and tick-borne encephalitis or Langat virus capsid/pre-membrane signal sequences, or a variant thereof. In various examples, the sequences encoding the capsid/pre-membrane signal sequence of the flavivirus genome include yellow fever virus sequences in the 5' region and tick-borne encephalitis or Langat virus sequences in the 3' region.

Further, the disclosure~~invention~~ includes replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), wherein the flavivirus genome includes West Nile virus sequences in which sequences encoding pre-membrane and envelope proteins are replaced with sequences encoding pre-membrane and envelope

proteins of a tick-borne encephalitis or a Langat virus, and the sequences encoding the signal sequence between the capsid and pre-membrane proteins of the flavivirus genome include sequences encoding a tick-borne encephalitis or a Langat virus capsid/pre-membrane signal sequence, or a variant thereof.

In addition, the disclosure~~invention~~ includes replication-deficient pseudoinfectious flaviviruses including a flavivirus genome including one or more deletions or mutations in nucleotide sequences encoding one or more proteins selected from the group consisting of capsid (C), pre-membrane (prM), envelope (E), non-structural protein 1 (NS1), non-structural protein 3 (NS3), and non-structural protein 5 (NS5), wherein any capsid (C) and non-structural (NS) proteins in the flavivirus genome are from Langat virus and any pre-membrane (prM) and envelope (E) proteins are from a tick-borne encephalitis virus.

By "replication-deficient pseudoinfectious flavivirus" or "PIV" is meant a flavivirus that is replication-deficient due to a deletion or mutation in the flavivirus genome. The deletion or mutation can be, for example, a deletion of a large sequence, such as most of the capsid protein, as described herein (with the cyclization sequence remaining; see below). In other examples, sequences encoding different proteins (e.g., prM, E, NS1, NS3, and/or NS5; see below) or combinations of proteins (e.g., prM-E or C-prM-E) are deleted. This type of deletion may be advantageous if the PIV is to be used a vector to deliver a heterologous immunogen, as the deletion can permit insertion of sequences that may be, for example, at least up to the size of the deleted sequence. In other examples, the mutation can be, for example, a point mutation, provided that it results in replication deficiency, as discussed above. Because of the deletion or mutation, the genome does not encode all proteins necessary to produce a full flavivirus particle. The missing sequences can be provided in trans by a complementing cell line that is engineered to express the missing sequence (e.g., by use of a replicon; s-PIV; see below), or by co-expression of two replication-deficient genomes in the same cell, where the two replication-deficient genomes, when considered together, encode all proteins necessary for production (d-PIV system; see below).

Upon introduction into cells that do not express complementing proteins, the genomes replicate and, in some instances, generate "virus-like particles," which are