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(54) Title: LIVE ATTENUATED INFLUENZA B VIRUS COMPOSITIONS METHODS OF MAKING AND USING THEREOF

(57) Abstract: The NS1 protein of influenza virus is a key virulent element with multi-functional roles in virus replication and acts as a strong interferon (IFN) antagonist. A live attenuated virus (LAIV) is provided using a master backbone, which contains the influenza B (HK8038) virus and includes a deletion of the NS1 coding region (DelNS1). The LAIV is based on novel adaptive mutations, which support DelNS1 influenza B live attenuated virus (LAIV) replication in vaccine producing cells. DelNS1 influenza B LAIV shows spontaneous cold adaption with preference to grow at 30-33°C but restriction at 37-39°C. The LAIV can be used to protect a subject in need thereof, against a lethal challenge of antigenic distant influenza B viruses. DelNS1 LAIV with adaptive mutations for growing in vaccine producing systems is an important strategy for making highly attenuated and immunogenic live attenuated influenza vaccines with the ability to induce broad cross protective immunity for seasonal influenza.



LIVE ATTENUATED INFLUENZA B VIRUS COMPOSITIONS METHODS OF MAKING AND USING THEREOF

FIELD OF THE INVENTION

5 The present invention is generally in the field of live attenuated influenza virus (LAIV) B and immunogenic compositions including LAIVB, and methods of using such compositions for inducing an immune response to antigen(s) encoded by the viral genome.

BACKGROUND OF THE INVENTION

10 The influenza viruses are classified into types A, B, and C on the basis of differences in viral nucleoprotein (NP) and matric protein. Type A viruses are further subdivided different subtypes according to their envelope glycoproteins with haemagglutinin (HA) or neuraminidase (NA) activity. There are 18 different hemagglutinin subtypes and 11 different neuraminidase subtypes. (H1 through H18 and
15 N1 through N11 respectively.) Influenza B viruses are not divided into subtypes, but can be further broken down into lineages and strains. Currently circulating influenza B viruses belong to one of two lineages: B/Yamagata and B/Victoria. Influenza B and C viruses mainly affect humans, whereas influenza A viruses infect a range of mammalian and avian species. Only type A and B cause human disease of any concern. Influenza
20 occurs all over the world, with an annual global attack rate estimated at 5 – 10% in adults and 20 – 30% in children (Thompson, et al., *JAMA* 292, 1333-1340 (2004); World Health Organization Influenza (seasonal) fact sheet. *World Health Organization (online)* <http://www.who.int/mediacentre/factsheets/fs211/en> (2014)), leading 2 to 5 million severe disease and as many as 650,000 cases are fatal each year (Iuliano et al., *Lancet*
25 391, 1285-1300 (2018); World Health Organization, 2016 Influenza (seasonal) fact sheet 211. www.who.int/mediacentre/factsheets/fs211/en) . Vaccination with flu vaccine is considered to be the most effective way to alleviate disease burden and mortality caused by seasonal influenza, as well as to prevent future pandemics in humans.

30 There are currently three types of inactivated vaccines available, the whole virus vaccines, split virus vaccines, and subunit vaccines. In split virus vaccines, the virus has been disrupted by a detergent. In subunit vaccines, HA and NA have been further purified by removal of other viral components. Live, attenuated influenza vaccines have been based on cold-adapted and temperature-sensitive variant vaccine virus strains that

replicate well in the nasopharynx of upper respiratory tract but poorly in the lower respiratory tract (Jin, et al. *Curr Top Microbiol Immunol* 386, 181-204 (2015)). Current licensed flu vaccines are either trivalent (three-component) or quadrivalent (four-component). Trivalent influenza vaccines composed of antigens derived from two influenza A, H1N1 and H3N2 subtype viruses, and either strain of influenza B virus from Victoria or Yamagata lineage based on surveillance data of World Health Origination (WHO) influenza network laboratories. A quadrivalent flu vaccine was first licensed in 2012 in the US and made available in the 2013-2014 flu season. Quadrivalent vaccines which contain antigens for protection against influenza A H1N1, H3N2 subtypes virus and both lineages of flu B viruses were recommended in 2013 by WHO, but leaving each country/state to decide to use either trivalent or quadrivalent vaccine. Flu vaccine strains are selected annually according to the data collected by the surveillance program from collaborating network laboratories of WHO for influenza vaccine. There is disparity of illness among humans caused by different strains of influenza virus, with influenza B more common in children and H3N2 more among the elderly (Caini, et al., *BMC Infect Dis* **18**, 269 (2018)). While it was previously thought that influenza B virus mainly causes diseases for older children, it has been noticed that there is a gradually change of the epidemic pattern of influenza B virus with diseases burden in all age groups since 2005 (Glezen, et al., *Am J Public Health* **103**, e43-51 (2013)). Since influenza B virus is only circulating in humans, an effective vaccine influenza B virus may eventually achieve eradication of flu B virus from humans. Like the circulation of H1N1 and H3N2 subtypes, two antigenic distinct flu B virus, Yamagata and Victoria lineages, circulate in humans alternatively, with the trend difficult to predict correctly each year. Current seasonal flu vaccines are suboptimal for the variable nature of circulating flu viruses each season. Further, although live attenuated influenza virus (LAIV) vaccine has advantages because it induces both humoral and cellular immunity, the current available LAIV vaccine is only licensed for use in 2-49 age group, due to the safety concern in young children and uncertain efficacy in the older age group. The NS1 protein of influenza virus has multiple functions and is a determinant of virulence, making its deletion an attractive target strategy for live vaccines. However, deletion of NS1 severely affects virus replication, making it difficult to produce high titers of attenuated virus for vaccine applications.

Talon, et al., *PNAS*, 97(8):4309-1314 (2000) disclose the growth characteristics of influenza B/201 and B/234 viral variants. Influenza B/201 and influenza B/AWBY/234 (B/234) viruses are laboratory variants of influenza B/Yamagata/1/73 that contain alterations in the NS1 ORF as compared with the NS1 ORF of wild-type influenza B/Yamagata. Nucleotide deletions in the NS gene of either B/201 or B/234 create frame shifts that result in the generation of shortened NS1 proteins. The influenza B/201 and B/234 viral NS1 proteins contain, respectively, 110 and 89 amino acids corresponding to the N terminus of the B/Yamagata viral NS1 protein. Both B/201 and B/234 viruses grow in 6-day-old eggs albeit to lower titers than B/Yamagata virus. However, whereas B/Yamagata grew equally well in 8-day-old eggs, growth of the other two viruses was markedly reduced in embryos of this age. Talon, et al., *PNAS*, 97(8):4309-1314 (2000).

Hai, et al., *J. Virol.*, 82(21):10580-10590 (2008) discloses influenza B virus truncated mutants of influenza B/Yamagata/88. For the generation of the different NS1 truncation mutants, a total of three influenza B/Yamagata/88 virus NS1 mutants, deltaNS1, NS1-80, and NS1-110, were constructed by using the pDZ plasmid. The coding region of the influenza B/Yamagata/88 virus NS1 gene is 846 nucleotides long (corresponding to 282 amino acids). Deletions and sequential truncations corresponding to the NS1 C terminus were made in this segment, resulting in three constructs encoding amino acids 1 to 16 (deltaNS1), 1 to 80 (NS1-80), or 1 to 110 (NS1-110) of the NS1 protein. The open reading frame of the NEP (nuclear export protein) was not altered in these constructs. According to Hai et al., the mutant viruses exhibited lower peak titers than the rWT virus in IFN-deficient Vero cells as well as in MDCK cells, although the deltaNS1 virus did grow more efficiently in Vero cells than in MDCK cells.

Pica et al., *J. Virol.*, 86(19):10293-10301 (2012) discloses recombinant viruses expressed either the full-length NS1 protein (wild-type [WT] PR8) or the first 126 amino acids (NS1-126).

Novel strategies to develop more effective vaccine against influenza B virus with properties to provide broad cross protective activity are necessary.

It is an object of the present invention to provide a safer and more effective live attenuated influenza B vaccine.

It is also an object of the present invention to provide methods of generating mutant influenza B viruses with improved ability to replication in influenza vaccine producing systems.

It is a further object of the present invention to provide methods of replicating live attenuated influenza B virus with high titre values useful for vaccine applications.

It is a further object of the present invention to provide methods of eliciting an immune response against an influenza virus B in a mammal.

5 Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

10 Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

SUMMARY OF THE INVENTION

15 A Live attenuated influenza virus (LAIV) B "LAIVB") from Victoria lineage is provided, with a deletion of the viral virulence element, the NS1 (non-structural protein 1), LAVIB/DelNS1. The LAIVB/DelNS1 additionally includes adaptive nucleotide mutations (AM) in segments 3 and 5-7 as follows: PA(T210C), NA T(1424C), NP(C182T) and M (A281G). The mutations are highlighted in the DNA sequences. The
20 LAIB/DelNS1 with adaptive mutations is referred to herein as AM/LAVIB/DelNS1. The AM/LAVIB/DelNS1preferably, is not able to replicate in interferon-competent cells, for example, A549 cells, and preferably replicates at low temperatures such as temperatures below 37 °C, more preferably between 30 and 33 °C and most preferably, at about 33 °C. In a particularly preferred embodiment, the disclosed LAIVB is characterized in that it
25 replicated poorly in MDCK cells at 37 °C, when compared to its replication at 33 °C in the MDCK cells. In a particularly preferred embodiment, the mutated LAIVB/DelLNS1 is able to replicate at levels comparable to wild type influenza virus of the same strain, in a vaccine producing system for example, eggs, or MDCK cells. For example, the LAIVB/DelLNS1 is able to replicate at levels $> 10^7$ plaque forming units (pfu/ml) for
30 example, between 10^7 - 10^8 pfu/ml.

Also disclosed are methods for making LAIVB with a deletion of the viral virulence element, the NS1 protein and adaptive mutations that allows growth of the mutated strain in vaccine producing systems such as eggs and MDCK cells (i.e.,

AM/LAVIB/DelNS1). Preferred virus strains include B/Yamagata and B/Victoria. The preferred adaptive mutations are PA(T210C), NA T(1424C), NP(C182T) and M (A281G) mutations in a Victoria strain. The methods include (a) generating influenza B virus with deletion of the coding region of the NS1 coding region by transfecting the DelNS1 influenza B virus into one or more vaccine producing cells (b) rescuing LAVIB/DelNS1 and (c) passaging rescued virus in one or more vaccine producing cells until viral titer is stabilized, to obtain AM/LAVIB/DelNS1.

The disclosed methods preferably include reverse genetics. In particularly preferred embodiments, plasmids containing the deleted NS1 segment (DelNS1) and the other seven genome segments derived from an influenza B virus strain are transfected into 293T/MDCK cell mixture. Rescued virus is passaged in MDCK cells until virus titer is stabilized, with virus titer maintains without meaningful change for three consecutive passages. As used herein, without meaningful change refers to changes including no change or no statistically significant change. Whole sequence of growth-adapted DelNS1 influenza B virus was analyzed and acquired mutations were identified as presented in Fig. 1B. The virus is sequenced to determine adaptive mutations.

Pharmaceutical compositions are also provided. The pharmaceutical compositions include the disclosed immunogenic AM/LAVIB/DelNS1 produced according to the disclosed methods. The pharmaceutical compositions typically include an effective amount of a virus to induce an immune response in subject in need thereof when administered to the subject. The pharmaceutical compositions can include additional agents, for example adjuvants to enhance the immune response. In some embodiments, the pharmaceutical compositions do not include an adjuvant. In one embodiment, the composition include an effective mount of a AM/LAVIB/DelNS1 from influenza of the Victoria lineage, with an NS1 deletion and mutations in segments 3, and 5-7 as follows: PA(T210C), NA T(1424C), NP(C182T) and M (A281G).

Methods of treating a subject in need thereof by administering the pharmaceutical composition to the subject are also provided. The methods can be vaccine protocols. Thus, in some embodiments, the subject is administered the composition to provide prophylactic or therapeutic protection against the virus itself (e.g., autologous antigen(s)) alone or in combination with protection against an infection from another virus, preferably, another influenza virus A or B strain. The disclosed AM/LAVIB/DelNS1 generated according to the methods disclosed here are administered to a mammal in need

thereof by subcutaneous (s.c.), intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), oral, or intranasal administration; or by injection or by inhalation. In other aspects, the strain is administered intranasally. The AM/LAVIB/DelNS1 is administered to a mammal in need of protective immunity against the influenza infection.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B show generation and characterization of DelNS1 influenza B live attenuated virus vaccine. **FIG. 1A** is an illustration of generation of DelNS1-8308B influenza B virus by reverse genetics. pHW2000 plasmids containing the DelNS1 segment and the other seven genome segments derived from B/Hong Kong/8038/2011 (Victoria) virus were transfected into 293T/MDCK cell mixture. Rescued virus was passaged in MDCK cells until virus titer was stabilized. **Fig. 1B.** shows identified adaptive mutations in growth adapted DelNS1-8308B influenza B virus. Four mutations in PA(T210C), NA T(1424C), NP(C182T) and M (A281G) were identified.

FIGS. 2A-2D show growth kinetics of DelNS1 virus were estimated in MDCK (**FIGS. 2A and 2B**) and A549 (**FIGS. 2C and 2D**) cells at 37°C (**FIGS. 2A and 2C**) and 33°C degrees respectively (**FIGS. 2B and 2D**). DelNS1 flu B virus is not able to replicate in A549 cells and poorly replicate in MDCK cells at 37°C degree. However, DelNS1 flu B virus is able to replicate at levels comparable to wild type flu B virus at 33°C in MDCK cells.

FIGS. 3A and 3B show protection following lethal challenge in mouse with DelNS1 Flu B vaccine, measured in terms of body weight following vaccination. Mice were vaccinated with DelNS1 Flu B vaccine (Victoria lineage, 2×10^6 pfu) ONCE through nasal drop. After three weeks, vaccinated mice were subsequently challenged with either one of mouse adapted flu B viruses, B/Florida/4/2006 (Yamagata lineage, 5×10^5 TCID₅₀) (**FIG. 3A**) or B/Brisbane/60/ 2008 (Victoria lineage, 5×10^6 TCID₅₀) (**FIG. 3B**). Body weight and survival rate of the mice were recorded for 2 weeks. **FIG. 3C and 3D** show percent survival of mice vaccinated with DelNS1 Flu B vaccine or PBS and challenged with B/Florida/4/2006 (Fig. 3C) or B/Brisbane/60/ 2008 (Fig. 3D).

FIGS. 4A and 4B shows a comparison of viral titers in lungs of mice immunized with DelNS1 influenza B live attenuated virus vaccine and control (PBS mock vaccinated) on day 3 post infection of influenza B mouse adapted viruses, B/Florida/4/2006 (**FIG. 4A**) or B/Brisbane/60/ 2008 (**FIG. 4B**).

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

Materials

Disclosed are materials, compositions, and components that can be used for, can
5 be used in conjunction with, can be used in preparation for, or are products of the
disclosed method and compositions. These and other materials are disclosed herein, and
it is understood that when combinations, subsets, interactions, groups, etc. of these
materials are disclosed that while specific reference of each various individual and
collective combinations and permutation of these compounds may not be explicitly
10 disclosed, each is specifically contemplated and described herein. For example, if an
aptamer is disclosed and discussed and a number of modifications that can be made to a
number of molecules or compositions including the aptamer are discussed, each and
every combination and permutation of the aptamer and the modifications that are
possible are specifically contemplated unless specifically indicated to the contrary. Thus,
15 if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and
F and an example of a combination molecule, A-D is disclosed, then even if each is not
individually recited, each is individually and collectively contemplated. Thus, in this
example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are
specifically contemplated and should be considered disclosed from disclosure of A, B,
20 and C; D, E, and F; and the example combination A-D. Likewise, any subset or
combination of these is also specifically contemplated and disclosed. Thus, for example,
the sub-group of A-E, B-F, and C-E are specifically contemplated and should be
considered disclosed from disclosure of A, B, and C; D, E, and F; and the example
combination A-D. Further, each of the materials, compositions, components, etc.
25 contemplated and disclosed as above can also be specifically and independently included
or excluded from any group, subgroup, list, set, etc. of such materials. These concepts
apply to all aspects of this application including, but not limited to, steps in methods of
making and using the disclosed compositions. Thus, if there are a variety of additional
steps that can be performed it is understood that each of these additional steps can be
30 performed with any specific embodiment or combination of embodiments of the
disclosed methods, and that each such combination is specifically contemplated and
should be considered disclosed.

As used herein, the term "adjuvant" refers to a compound or mixture that enhances an immune response.

As used herein, "attenuated" refers to refers to procedures that weaken an agent of disease (a pathogen). An attenuated virus is a weakened, less vigorous virus. A

5 vaccine against a viral disease can be made from an attenuated, less virulent strain of the virus, a virus capable of stimulating an immune response and creating immunity but not causing illness or less severe illness. Attenuation can be achieved by chemical treatment of the pathogen, through radiation, or by genetic modification, using methods known to those skilled in the art. Attenuation may result in decreased proliferation, attachment to
10 host cells, or decreased production or strength of toxins.

As used herein, "autologous" means derived from self.

The term "child" is meant to be a person or a mammal between 0 months and 18 years of age and "young child" refers to a child < 5 yrs. old.

As used herein, the term "effective amount" or "therapeutically effective amount"
15 means a dosage sufficient to treat, inhibit, or alleviate one or more symptoms of a disease state being treated or to otherwise provide a desired pharmacologic effect. The precise dosage will vary according to a variety of factors such as subject-dependent variables (e.g., age, immune system health, etc.), the disease, and the age of the subject.

As used herein, the term "gene" refers to a nucleic acid (e.g., DNA or RNA)
20 sequence that including coding sequences necessary for the production of a polypeptide, RNA (e.g., including but not limited to, mRNA, tRNA and rRNA) or precursor. The polypeptide, RNA, or precursor can be encoded by a full length coding sequence or by any portion thereof. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends for a
25 distance of about 1 kb on either end such that the gene corresponds to the length of the full-length mRNA. The term "gene" encompasses both cDNA and genomic forms of a gene, which may be made of DNA, or RNA. A genomic form or clone of a gene may contain the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are
30 transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA

functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

The term “immunogenic composition” or “composition” means that the composition can induce an immune response and is therefore antigenic. By “immune response” means any reaction by the immune system. These reactions include the alteration in the activity of an organism's immune system in response to an antigen and can involve, for example, antibody production, induction of cell-mediated immunity, complement activation, or development of immunological tolerance.

The term “nasal administration” refers to any form of administration whereby an active ingredient is propelled or otherwise introduced into the nasal passages of a subject so that it contacts the respiratory epithelium of the nasal cavity, from which it is absorbed into the systemic circulation. Nasal administration can also involve contacting the olfactory epithelium, which is located at the top of the nasal cavity between the central nasal septum and the lateral wall of each main nasal passage. The region of the nasal cavity immediately surrounding the olfactory epithelium is free of airflow. Thus, specialized methods must typically be employed to achieve significant absorption across the olfactory epithelium.

The terms “oral”, “enteral”, “enterally”, “orally”, “non-parenteral”, “non-parenterally”, and the like, refer to administration of a compound or composition to an individual by a route or mode along the alimentary canal. Examples of “oral” routes of administration of a composition include, without limitation, swallowing liquid or solid forms of a vaccine composition from the mouth, administration of a vaccine composition through a nasojejunal or gastrostomy tube, intraduodenal administration of a vaccine composition, and rectal administration, e.g., using suppositories that release a live bacterial vaccine strain described herein.

The term “mammal” as used herein includes both humans and non-humans and include but is not limited to humans, non-human primates, canines, felines, murines, bovines, equines, and porcines.

The term “topical administration” refers to the application of a pharmaceutical agent to the external surface of the skin or the mucous membranes (including the surface membranes of the nose, lungs and mouth), such that the agent crosses the external surface of the skin or mucous membrane and enters the underlying tissues. Topical administration can result in a limited distribution of the agent to the skin and surrounding

tissues or, when the agent is removed from the treatment area by the bloodstream, systemic distribution of the agent. In a preferred form, the agent is delivered by transdermal delivery, e.g., using a transdermal patch. Transdermal delivery refers to the diffusion of an agent across the skin (stratum corneum and epidermis), which acts as a barrier few agents are able to penetrate. In contrast, the dermis is permeable to absorption of many solutes and drugs, and topical administration therefor occurs more readily through skin which is abraded or otherwise stripped of the epidermis to expose the dermis. Absorption through intact skin can be enhanced by combining the active agent with an oily vehicle (e.g., creams, emollients, penetration enhancers, and the like, as described, e.g., in Remington's Pharmaceutical Sciences, current edition, Gennaro et al., eds.) prior to application to the skin (a process known as inunction).

As used herein, the term "peptide" refers to a class of compounds composed of amino acids chemically bound together. In general, the amino acids are chemically bound together via amide linkages (CONH); however, the amino acids may be bound together by other chemical bonds known in the art. For example, the amino acids may be bound by amine linkages. Peptide as used herein includes oligomers of amino acids and small and large peptides, including polypeptides.

As used herein "recombinant DNA" refers to DNA molecules that is extracted from different sources and chemically joined together; for example DNA including a gene from one source may be recombined with DNA from another source. Recombinant DNA can be all heterologous DNA or a combination of homologous and heterologous DNA. The recombinant DNA can be integrated into and expressed from a cell's chromosome, or can be expressed for an extra-chromosomal array such as a plasmid.

As used herein, a "variant," "mutant," or "mutated" polynucleotide or polypeptide contains at least one polynucleotide or polypeptide sequence alteration as compared to the polynucleotide or polypeptide sequence of the corresponding wild-type or parent polynucleotide or polypeptide. Mutations may be natural, deliberate, or accidental. Mutations include substitutions, deletions, and insertions.

II. COMPOSITIONS

Immunogenic compositions including live attenuated influenza B (LAIVB) virus are provided. The compositions are immunogenic in that they can be used to elicit an immune response against one or more antigens encoded by the LAIVB. The LAIVB has improved safety due to deletion of the coding region of the NS1 segment (DelNS1) and

adaptive mutations (AM) which improve its growth in vaccine producing systems. Influenza B viruses with these combinations of mutations are referred to herein as AM/LAVIB/DelNS1. The examples demonstrate that AM/LAVIB/DelNS1 virus is not virulent to mice, even when challenged with the highest dose of virus (Figure 3A and 3B). The AM/LAVIB/DelNS1 can be included in a formulation for administration, in a carrier, and in some embodiments, in combination with an adjuvant. The adjuvant can serve as the carrier. In some embodiments, immunogenic compositions containing the disclosed AM/LAVIB/DelNS1 do not include an adjuvant.

A. Live Attenuated Virus

The influenza B virus genomes each comprise eight negative-sense, single-stranded viral RNA (vRNA) segments. The smallest vRNA segment of both viruses encodes the non-structural NS1 protein. Influenza B virus expresses from an unspliced transcript of the viral NS segment a 281-amino-acid nonstructural protein termed NS1-B. The disclosed LAIVB preferably include a complete deletion of the coding region for NS1 from the LAIVB genome. The AM/LAVIB/DelNS1 can be of the Yamagata or Victoria lineage. During 1988-1989 two highly distinct antigenic variants of influenza type B were recognized in hemagglutination-inhibition tests with post infection ferret serum. These viruses were antigenically related to either B/Victoria/2/87, the most recent reference strain, or B/Yamagata/16/88, a variant that was isolated in Japan in May 1988. All influenza B viruses isolated in the United States during an epidemic in the winter of 1988-1989 were antigenically related to B/Victoria/2/87. Different strains of influenza B virus are disclosed in the Influenza Research Database. Zhang, et al., *Nucleic Acids Research*, Volume 45(D1): D466–D474, 2017.

The eight segments of influenza B viruses (and are numbered in order of decreasing length (TABLE 1). Segments 1, 3, 4, and 5 encode just one protein per segment: the PB2, PA, HA and NP proteins.

Table 1. Segments of Influenza B virus and their encoded proteins

Segment	Encoded Protein	Protein Function
1 (SEQ ID NO: 1)	PB2	Polymerase subunit; mRNA cap recognition
2 (SEQ ID NO: 2)	PB1	Polymerase subunit; RNA elongation, endonuclease activity
3 (SEQ ID NO: 3)	PA	Polymerase subunit; protease activity
4 (SEQ ID NO: 4)	HA	Surface glycoprotein; major antigen, receptor binding and fusion activities
5 (SEQ ID NO: 5)	NP	RNA binding protein; nuclear import regulation
6 (SEQ ID NO: 6)	NA NB	Surface glycoprotein; sialidase activity, virus release an integral membrane protein corresponding to the influenza A virus

		M2 protein
7 (SEQ ID NO: 7)	M1	Matrix protein; vRNP interaction, RNA nuclear export regulation, viral budding
8 (SEQ ID NO: 8)	NS1	Interferon antagonist protein; regulation of host gene expression
	NEP/NS2	Nuclear export of RNA

All influenza viruses encode the polymerase subunit PB1 on segment 2.

The mutated influenza B virus disclosed herein includes an NS1 deletion, and the following mutations: PA(T210C), NA(T1424C), NP (C182T) and M (A281G). The disclosed LAIV B can be readily distinguished from viruses described in U.S. Patent No. 8592196.

Similar mutations can be introduced into the respective segments for other influenza B virus; the segment sequences which are publicly disclosed. For example, the GenBank accession number for various influenza B virus segment 7 nucleotide sequence are: DQ792908.1 (Influenza B virus (B/Lee/40) cold-adapted M1 protein (M1) and BM2 protein (BM2) genes, complete cds); M20175.1 (Influenza B/Ann Arbor/1/66 (cold-adapted) membrane protein M1 (seg 7) RNA, complete cds); CY018758.1 (Influenza B virus (B/Victoria/02/1987) segment 7, complete sequence); CY018686.1 (Influenza B virus (B/Hong Kong/1434/2002) segment 7, complete sequence).

B. Adjuvants

The disclosed LAIV can be administered in conjunction with other immunoregulatory agents, including adjuvants. Useful adjuvants but are not limited to, one or more set forth below:

Mineral Containing Adjuvant Compositions include mineral salts, such as aluminum salts and calcium salts. Exemplary mineral salts include hydroxides (e.g., oxyhydroxides), phosphates (e.g., hydroxyphosphates, orthophosphates), sulfates, and the like or mixtures of different mineral compounds (e.g., a mixture of a phosphate and a hydroxide adjuvant, optionally with an excess of the phosphate), with the compounds taking any suitable form (e.g., gel, crystalline, amorphous, and the like), and with adsorption to the salt(s) being preferred. The mineral containing compositions can also be formulated as a particle of metal salt (WO/0023105). Aluminum salts can be included in compositions of the invention such that the dose of Al^{3+} is between 0.2 and 1.0 mg per dose.

Oil-Emulsion Adjuvants suitable for use as adjuvants in the invention can include squalene-water emulsions, such as MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span

85, formulated into submicron particles using a microfluidizer). See, e.g., WO90/14837. and, Podda, *Vaccine* 19: 2673-2680, 2001. Additional adjuvants for use in the compositions are submicron oil-in-water emulsions. Examples of submicron oil-in-water emulsions for use herein include squalene/water emulsions optionally containing varying amounts of MTP-PE, such as a submicron oil-in-water emulsion containing 4-5% w/v squalene, 0.25-1.0% w/v Tween 80 (polyoxyethylsorbitan monooleate), and/or 0.25-1.0% Span 85 (sorbitan trioleate), and, optionally, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-s-n-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), for example, the submicron oil-in-water emulsion known as "MF59" (International Publication No. WO90/14837; U.S. Pat. Nos. 6,299,884 and 6,451,325, incorporated herein by reference in their entirety. MF59 can contain 4-5% w/v Squalene (e.g., 4.3%), 0.25-0.5% w/v Tween 80, and 0.5% w/v Span 85 and optionally contains various amounts of MTP-PE, formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, Mass.). For example, MTP-PE can be present in an amount of about 0-500 µg/dose, or 0-250 µg/dose, or 0-100 µg/dose. Submicron oil-in-water emulsions, methods of making the same and immunostimulating agents, such as muramyl peptides, for use in the compositions, are described in detail in International Publication No. WO90/14837 and U.S. Pat. Nos. 6,299,884 and 6,451,325.

Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) can also be used as adjuvants in the invention.

Saponin Adjuvant Formulations can also be used as adjuvants in the invention. Saponins are a heterologous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even flowers of a wide range of plant species. Saponin from the bark of the *Quillaia saponaria* Molina tree have been widely studied as adjuvants. Saponin can also be commercially obtained from *Smilax ornata* (sarsapilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations can include purified formulations, such as QS21, as well as lipid formulations, such as Immunostimulating Complexes (ISCOMs; see below). Saponin compositions have been purified using High Performance Thin Layer Chromatography (HPLC) and Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. A method

of production of QS21 is disclosed in U.S. Pat. No. 5,057,540. Saponin formulations can also comprise a sterol, such as cholesterol (*see* WO96/33739). Combinations of saponins and cholesterol can be used to form unique particles called ISCOMs.

ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. For example, an ISCOM can include one or more of Quil A, QHA and QHC. ISCOMs are described in EP0109942, WO96/11711, and WO96/33739. Optionally, the ISCOMS can be devoid of additional detergent. *See* WO00/07621. A description of the development of saponin based adjuvants can be found at Barr, et al., "ISCOMs and other saponin based adjuvants", *Advanced Drug Delivery Reviews* 32: 247-27, 1998. See also Sjolander, et al., "Uptake and adjuvant activity of orally delivered saponin and ISCOM vaccines", *Advanced Drug Delivery Reviews* 32: 321-338, 1998.

Virosomes and Virus Like Particles (VLPs) can also be used as adjuvants. These structures generally contain one or more proteins from a virus optionally combined or formulated with a phospholipid. They are generally non-pathogenic, non-replicating and generally do not contain any of the native viral genome. The viral proteins can be recombinantly produced or isolated from whole viruses. These viral proteins suitable for use in virosomes or VLPs include proteins derived from influenza virus (such as HA or NA), Hepatitis B virus (such as core or capsid proteins), Hepatitis E virus, measles virus, Sindbis virus, Rotavirus, Foot-and-Mouth Disease virus, Retrovirus, Norwalk virus, human Papilloma virus, HIV, RNA-phages, QB-phage (such as coat proteins), GA-phage, fr-phage, AP205 phage, and Ty (such as retrotransposon Ty protein pl).

Bacterial or Microbial Derivatives useful as adjuvants include: (i) Non-Toxic Derivatives of Enterobacterial Lipopolysaccharide (LPS); (ii) lipid derivatives, (iii) immunostimulatory oligonucleotides and ADP-Ribosylating Toxins and Detoxified Derivatives Thereof, (iv) ADP-Ribosylating Toxins and Detoxified Derivatives Thereof. Examples of Non-Toxic Derivatives of LPS Monophosphoryl lipid A (MPL) and 3-O-deacylated MPL (3 dMPL). 3 dMPL is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. An example of a "small particle" form of 3 De-O-acylated monophosphoryl lipid A is disclosed in EP 0 689 454. Such "small particles" of 3 dMPL are small enough to be sterile filtered through a 0.22 micron membrane (*see* EP 0 689 454). Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives e.g., RC-529 (Johnson

et al., *Bioorg Med Chem Lett*, 9: 2273-2278, 1999). Examples of lipid A derivatives can include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in Meraldi et al., "OM-174, a New Adjuvant with a Potential for Human Use, Induces a Protective Response with Administered with the Synthetic C-Terminal Fragment 242-310 from the circumsporozoite protein of *Plasmodium berghei*", *Vaccine* 21: 2485-2491, 2003; and Pajak, et al., "The Adjuvant OM-174 induces both the migration and maturation of murine dendritic cells in vivo", *Vaccine* 21: 836-842, 2003. Examples of immunostimulatory oligonucleotide sequences containing a CpG motif (a sequence containing an unmethylated cytosine followed by guanosine and linked by a phosphate bond). Bacterial double stranded RNA or oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. Optionally, the guanosine can be replaced with an analog such as 2'-deoxy-7-deazaguanosine. See Kandimalla, et al., "Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles", *Nucleic Acids Research* 31: 2393-2400, 2003; WO02/26757 and WO99/62923 for examples of analog substitutions. The adjuvant effect of CpG oligonucleotides is further discussed in Krieg, *Nature Medicine* (2003) 9(7): 831-835; McCluskie, et al., *FEMS Immunology and Medical Microbiology* (2002) 32:179-185; WO98/40100; U.S. Pat. No. 6,207,646; U.S. Pat. No. 6,239,116 and U.S. Pat. No. 6,429,199. The CpG sequence can be directed to Toll-like receptor (TLR9), such as the motif GTCGTT or TTCGTT. See Kandimalla, et al., "Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic CpG DNAs", *Biochemical Society Transactions* (2003) 31 (part 3): 654-658. The CpG sequence can be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it can be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in Blackwell, et al., *J. Immunol.* 170: 4061-4068, 2003; Krieg, *TRENDS in Immunology* 23: 64-65, 2002, and WO01/95935. In some aspects, the CpG oligonucleotide can be constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences can be attached at their 3' ends to form "immunomers". See, for example, Kandimalla, et al., *BBRC* 306: 948-95,

2003; Kandimalla, et al., *Biochemical Society Transactions* 31: 664-658, 2003; Bhagat et al., " *BBRC* 300: 853-861, 2003, and WO03/035836. Bacterial ADP-ribosylating toxins and detoxified derivatives thereof can be used as adjuvants in the invention. For example, the toxin can be derived from *E. coli* (i.e., *E. coli* heat labile enterotoxin (LT)), cholera (CT), or pertussis (PTX). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in WO95/17211 and as parenteral adjuvants in WO98/42375. In some aspects, the adjuvant can be a detoxified LT mutant such as LT-K63, LT-R72, and LTR192G. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in the following references, each of which is specifically incorporated by reference herein in their entirety: Beignon, et al., *Infection and Immunity* 70: 3012-3019, 2002; Pizza, et al., *Vaccine* 19: 2534-2541, 2001; Pizza, et al., *Int. J. Med. Microbiol* 290: 455-461, 2003; Scharton-Kersten et al., *Infection and Immunity* 68: 5306-5313, 2000; Ryan et al., *Infection and Immunity* 67: 6270-6280, 2003; Partidos et al., *Immunol. Lett.* 67: 09-216, 1999; Peppoloni et al., *Vaccines* 2: 285-293, 2003; and Pine et al., *J. Control Release* 85: 263-270, 2002.

Bioadhesives and mucoadhesives can also be used as adjuvants in the invention. Suitable bioadhesives can include esterified hyaluronic acid microspheres (Singh et al., *J. Cont. Rel.* 70:267-276, 2001) or mucoadhesives such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrrolidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof can also be used as adjuvants in the invention disclosed for example in WO99/27960.

Adjuvant Microparticles: Microparticles can also be used as adjuvants. Microparticles (i.e., a particle of about 100 nm to about 150 μ m in diameter, or 200 nm to about 30 μ m in diameter, or about 500 nm to about 10 μ m in diameter) formed from materials that are biodegradable and/or non-toxic (e.g., a poly(alpha-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, and the like), with poly(lactide-co-glycolide) are envisioned, optionally treated to have a negatively-charged surface (e.g., with SDS) or a positively-charged surface (e.g., with a cationic detergent, such as CTAB).

Examples of liposome formulations suitable for use as adjuvants are described in U.S. Pat. No. 6,090,406, U.S. Pat. No. 5,916,588, and EP 0 626 169.

Additional adjuvants include polyoxyethylene ethers and polyoxyethylene esters. WO99/52549. Such formulations can further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol (WO 01/21207) as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol (WO 01/21152). In some aspects, polyoxyethylene ethers can include: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, or polyoxyethylene-23-lauryl ether.

PCPP formulations for use as adjuvants are described, for example, in Andrianov et al., *Biomaterials* 19: 109-115, 1998.1998. Examples of muramyl peptides suitable for use as adjuvants in the invention can include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-1-alanyl-d-isoglutamine (nor-MDP), and N-acetylmuramyl-1-alanyl-d-isoglutaminyl-1-alanine-2-(1'-2'-dipalmitoyl-s-n-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE). Examples of imidazoquinolone compounds suitable for use as adjuvants in the invention can include Imiquimod and its homologues, described further in Stanley, "Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential" *Clin Exp Dermatol* 27: 571-577, 2002 and Jones, "Resiquimod 3M", *Curr Opin Investig Drugs* 4: 214-218, 2003. Human immunomodulators suitable for use as adjuvants in the invention can include cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, and the like), interferons (e.g., interferon-gamma), macrophage colony stimulating factor, and tumor necrosis factor.

Adjuvant Combinations: The adjuvants are used in come preferred embodiments as combinations. For example, adjuvant compositions can include: a saponin and an oil-in-water emulsion (WO99/11241); a saponin (e.g., QS21)+a non-toxic LPS derivative (e.g., 3 dMPL) (see WO94/00153); a saponin (e.g., QS21)+a non-toxic LPS derivative (e.g., 3 dMPL)+a cholesterol; a saponin (e.g., QS21)+3 dMPL+IL-12 (optionally+a sterol) (WO98/57659); combinations of 3 dMPL with, for example, QS21 and/or oil-in-water emulsions (See European patent applications 0835318, 0735898 and 0761231); SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion. Ribi adjuvant system (RAS), (Ribi Immunochem) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell

wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (Detox); and one or more mineral salts (such as an aluminum salt)+a non-toxic derivative of LPS (such as 3 dPML).

5 Aluminum salts and MF59 are examples of adjuvants for use with injectable influenza vaccines. Bacterial toxins and bioadhesives are examples of adjuvants for use with mucosally-delivered vaccines, such as nasal vaccines. All adjuvants noted above and others as generally known in the art to one of ordinary skill can be formulated for intranasal administration using techniques well known in the art.

10 C. Formulations and carriers

The composition of the invention can be formulated in pharmaceutical compositions. These compositions can comprise, in addition to one or more of the strains, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer, or other materials well known to those skilled in the art. Such materials should typically be non-toxic and should not typically interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material can depend on the route of administration, e.g., oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, or intraperitoneal routes.

Pharmaceutical compositions for oral administration can be in tablet, capsule, powder or liquid form. A tablet can include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil, or synthetic oil. Physiological saline solution, dextrose, or other saccharide solution or glycols such as ethylene glycol, propylene glycol, or polyethylene glycol can be included. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition (e.g., immunogenic or vaccine formulation) is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, ethanol and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. The formulation should be selected according to the mode of administration.

For intravenous, cutaneous, or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous

solution which is pyrogen-free and has suitable pH, isotonicity, and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, or Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants, and/or other additives can be included, as required.

Administration is preferably in a "therapeutically effective amount" or "prophylactically effective amount" (as the case can be, although prophylaxis can be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of disease being treated. Prescription of treatment, e.g., decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in the latest edition of Remington's Pharmaceutical Science, Mack Publishing Company, Easton, Pa. ("Remington's").

III. METHODS OF MAKING

A protocol to engineer AM/LAVIB/DelNS1 virus is provided. The protocol includes (a) generating influenza B virus with the coding region of the NS1 gene removed from its genome, by transfecting the eight plasmids, preferably pHW2000 plasmids, containing the genome of DelNS1 influenza B virus including a plasmid to express NS1 protein, into one or more vaccine producing cells with (b) rescuing LAVIB/DelNS1 virus and (c) passaging rescued virus into one or more vaccine producing cells at 33°C and 37°C respectively until viral titer is stabilized, for example, when the virus titer remains unchanged for three consecutive passages, to obtain AM/LAVIB/DelNS1 and (c) analyzing for the presence of desired mutations.

Whole sequence of growth-adapted DelNS1 influenza B virus was analyzed and compared to the sequence of parental strain, acquired mutations were identified. The coding region of the NS1 gene is removed from viral genome without affecting the expression of other essential genes for virus replication (illustrated in **Figure 1A**). In a preferred embodiment, the virus in step b is cultured in a mixture of two or more cells, for example, 293T/MDCK cells. The disclosed methods for making LAIVB result in a deletion of the viral virulence element, the NS1 protein and an adaptive mutation which

allows growth of the mutated strain in vaccine producing systems such as eggs and MDCK cells. The preferred adaptive mutations are PA(T210C), NA T(1424C), NP(C182T) and M (A281G) mutations. The disclosed methods preferably include reverse genetics. Plasmids containing the deleted NS1 segment (DelNS1) and the other seven genome segments derived from an influenza B virus strain are transfected into 293T/MDCK cells mixture. Preferred virus strains include B/Yamagata and B/Victoria Rescued virus, passaged in MDCK cells until virus titer is stabilized, until high levels of viral titer are obtained and remain unchanged for at least three consecutive passages. The virus is sequenced to determine adaptive mutations.

The coding region of the NS1 gene can be removed using methods known in the art. Methods to introduce targeted mutations into a genome or, in the context of virology, into a virus are subsumed under the term reverse genetics (RG) and are disclosed for example, in Hoffmann et al., *Proc Natl Acad Sci U S A*, 97(11):6108-13 Zheng et al., *J. Virol.* 89(20): 10273-85 and Dauber, et al., *J. Virol.*, 78(4):1865-1872 (2004), the materials and method of which are incorporated herein by reference. The method of generating influenza virus with the NS1 coding region deleted, as disclosed in Dauber et al. is generalized and summarized herein.

i. Generating influenza B virus with the coding region of the NS1 gene removed

a. RT-PCR and construction of plasmids

The RNeasy kit (Qiagen) can be used according to the manufacturer's protocol to extract viral RNA from a stock of influenza B virus. Eight reverse genetic plasmids pHW- PB2, pHW- PB1, pHW- PA, pHW- HA, pHW-NP, pHW-NA, pHW-M1, and pHW-NS1 (Table 1) are constructed by reverse transcriptase PCR (RT-PCR) amplification of single viral RNA segments and the resulting cDNAs cloned into the plasmid pHW2000. All eight plasmids can be used to generate the wild type influenza B virus. The primers used in the PCR amplification are listed in the following Table 2:

Table 2

Flub-pb1-lic-S (SEQ ID NO: 9)	CcgaagttggggggAGCAGAAGCGGAGC
Flub-pb1-lic-as (SEQ ID NO: 10)	GGCCGCCGGGTTATTAGTAGAAACACGAGC
Flub-pb2-lic-S (SEQ ID NO: 11)	CcgaagttggggggAGCAGAAGCGGAGC
Flub-pb2-lic-as (SEQ ID NO: 12)	GGCCGCCGGGTTATTAGTAGAAACACGAGC
Flub-pa-lic-S (SEQ ID NO: 13)	CcgaagttggggggAGCAGAAGCGGTGC
Flub-pa-lic-as (SEQ ID NO: 14)	GGCCGCCGGGTTATTAGTAGAAACACGTGC

Flub-ha-lic-S (SEQ ID NO: 15)	CcgaagtggggggAGCAGAAGCAGAGC
Flub-ha-lic-as (SEQ ID NO: 16)	GGCCGCCGGGTTATTAGTAGTAACAAGAGC
Flub-na-lic-S (SEQ ID NO: 17)	CcgaagtggggggAGCAGAAGCAGAGC
Flub-na-lic-as (SEQ ID NO: 18)	GGCCGCCGGGTTATTAGTAGTAACAAGAGC
Flub-np-lic-S (SEQ ID NO: 19)	CcgaagtggggggAGCAGAAGCACAGC
Flub-np-lic-as (SEQ ID NO: 20)	GGCCGCCGGGTTATTAGTAGAAACAACAGC
Flub-m-lic-S (SEQ ID NO: 21)	CcgaagtggggggAGCAGAAGCACGCACTT
Flub-m-lic-as (SEQ ID NO: 22)	GGCCGCCGGGTTATTAGTAGAAACAACGCACTT
Flub-ns-lic-S (SEQ ID NO: 23)	CcgaagtggggggAGCAGAAGCAGAGGATT
Flub-ns-lic-as (SEQ ID NO: 24)	GGCCGCCGGGTTATTAGTAGTAACAAGAGGATT
Flub-delns1-F (SEQ ID NO: 25)	CTCAAT TT GTGTTGTGGTC ATG
Flub-delns1-R (SEQ ID NO: 26)	TGGAGGATGAAGAAGATGGCCATCGGATCCTC

The coding region of the influenza B virus NS1 protein overlaps in part with the reading frame for the NEP/NS2 protein that is expressed from a spliced transcript of the viral NS gene segment. For the generation of NS1-deficient influenza B virus, we prepared a derivative of the NS reverse genetic plasmid termed pHW-Lee-ΔNS1-B, in which the sequences specifying the NS1 protein were deleted, while all NEP/NS2 coding sequences and the terminal noncoding regions were maintained. For example, for generation of the pHW- DelNS1-B plasmid, polyadenylated RNA is extracted from influenza B virus-infected MDCK cells by using the Oligotex direct mRNA Midi/Maxi Kit (Qiagen) and NS segment-specific primers used for RT-PCR amplification. Amplified NEP/NS2 fragment is purified with the QiaEx II gel extraction kit (Qiagen), digested with *Bsm*BI, and cloned into pHW2000.

These plasmids facilitate bidirectional transcription of negative-sense viral RNAs and positive-sense mRNAs since the cloned viral cDNAs are flanked upstream by a human RNA polymerase I promoter and downstream by an RNA polymerase II-specific promoter. In brief, the viral RNAs are first reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) by using a universal nine-nucleotide primer (UNI-9) that is complementary to the conserved 3' ends of all eight viral RNA segments. The RT reaction is performed for 60 min at 37°C, followed by 15 min at 70°C. Subsequently, single gene segments are amplified by PCR by using the *Pfu* Turbo Polymerase (Roche Diagnostics) and segment-specific primers carrying *Bsm*BI (PB1, PB2, PA, NA, M, and NS), *Bsp*MI (NP), or *Aar*I (HA) restriction site sequences at their 5' ends. pHW-NS-*Xho*I is a derivative of pHW--NS that is constructed with the QuikChange mutagenesis kit (Stratagene) by introducing a novel *Xho*I recognition site at

nucleotides 262 to 267 of the viral NS segment. The NS segment of the transfectant virus is engineered to carry an engineered genetic tag site such that the corresponding cDNA is susceptible to cleavage by the restriction endonuclease *XhoI*, thereby verifying the recombinant nature of the isolate.

5 **b. Transfection-mediated recovery of recombinant influenza B virus.**

To generate the recombinant influenza B wild-type virus the eight plasmids pHW - PB2, pHW-PB1, pHW-PA, pHW-HA, pHW-NP, pHW-NA, pHW-M, and pHW-NS-*XhoI* (0.5 µg each) are transfected into 10⁶ 293T cells in suspension with the Lipofectamine 2000 reagent (Invitrogen). At 72 h after transfection, the supernatant of transfected cells is inoculated into the allantoic cavities of 11-day-old chicken eggs to grow stocks of recombinant virus. Supernatants of transfected cells harboring mutant plasmid can be passaged into 6-day-old chicken eggs. The recovery of recombinant influenza B viruses can be verified by gel electrophoretic analyses of RT-PCR products representing the viral NS segments.

15 The plasmids are preferably transfected into a mixture of cells, for example, 293T/MDCK cells to grow stocks of recombinant virus. FIG. 1A The DelNS1 virus can be rescued by essentially the same procedure, except that pHW -NS-*XhoI* is replaced by pHW-ΔNS1-B and 0.5 µg of an expression vector for NS1, for example, pcDNA-NS1 added to the transfection mix. To construct plasmid pcDNA-NS1-B, the NS1 cDNA can be PCR amplified with pHW-Lee-NS as a template and cloned between the *HindIII/XhoI* sites of pcDNA3

25 The influenza B virus completely lacking the NS1 ORF disclosed in Dauber, et al. *J. Virol.*, 78:1865-1872 (2004) does not replicate efficiently in Vero cells (titres of 1.7-2.5*10² FFU/ml using an moi of 0.1 and no detectable titres at moi of 0,001, respectively). An influenza B NS1 deletion mutant consisting of the amino-terminal 16 aa is also highly attenuated in replication with maximum titres of approx. 10⁴ FFU/ml. (Hai et. al; Journal of Virology; November 2008, p. 10580-90

ii. Culturing rescued virus to obtain influenza B virus with comparable growth to wild type

30 Rescued DelNS1 virus is cultured in a virus-producing cell until virus titer is stabilized, with evidence that virus titer remains unchanged for at least three consecutive passages in MDCK cells and eggs. Referring to FIG. 1A, supernatant from the transfected cells after 72 hours is collected and passaged in MDCK cells.

A preferred cell for passaging is MDCK (Madin-Darby canine kidney) cells.

However, the cells used for the cultivation of viruses using a cultivation medium can be cells that can grow in vitro in synthetic media and can be used for the propagation of viruses. These can be for example BSC-1 cells, LLC-MK cells, CV-1 cells, CHO cells, COS cells, murine cells, human cells, HeLa cells, 293 cells, VERO cells, MDBK cells, MDOK cells, CRFK cells, RAF cells, TCMK cells, LLC-PK cells, PK15 cells, WI-38 cells, MRC-5 cells, T-FLY cells, BHK cells, SP2/0 cells, NS0, PerC6 (human retina cells), chicken embryo cells or derivatives, embryonated egg cells, embryonated chicken eggs or derivatives thereof.

The cultivation medium used for the production of viruses can be any medium known from prior art that is applicable for virus cultivation. Preferably the medium is a synthetic medium. This can be for example basal media as Modified Eagle's media MEM, minimum essential media MEM, Dulbecco's modified Eagle's media D-MEM, D-MEM-F12 media, William's E media, RPMI media and analogues and derivative thereof. These can also be specialty cell cultivation and virus growth media as VP-SFM, OptiPro™ SFM, AIM V® media, HyQ SFM4 MegaVir™, EX-CELL™ Vero SFM, EPISERF, ProVero, any 293 or CHO media and analogues and derivatives thereof. These media can be supplemented by any additive known from prior art that is applicable for cell and virus cultivation as for example animal sera and fractions or analogues thereof, amino acids, growth factors, hormones, buffers, trace elements, trypsin, sodium pyruvate, vitamins, L-glutamine and biological buffers. Preferable medium is OptiPRO™ SFM supplemented with L-glutamine and trypsin.

Thus, disclosed method includes culturing the virus in for an effective amount of time to obtain a stable viral titer. In preferred embodiment, the rescued virus is passaged in a virus-producing cell, for example, MDCK cells for a period of time until viral titre remains unchanged for 3 consecutive passages. This culture period can range from 10-50 passages, preferably, for over 20 passages at 33 °C . The time and conditions of culture result in adaptive mutations, which allows replication of the LAIVB in vaccine producing systems such as eggs or MDCK.

The examples demonstrate mutations in PA(T210C), NA(T1424C), NP (C182T) and M (A281G) which support DelNS1 flu B virus replication in chicken embryo eggs or the vaccine producing cell line, MDCK cells, for the viral strain tested (Figure 1B). The

DelNS1 flu B live attenuated virus with adaptive mutations is able to replicate to comparable level as wild type virus in vaccine producing systems, eggs or MDCK cells (Figure 2B). The DelNS1 live attenuated is not able to replicate in interferon-competent cells, A549. This a safety property to prevent virus shedding in vaccinated humans (Figure 2C AND 2D). The DelNS1 flu B virus prefers to replicate at lower temperature, such as 33⁰C, which is mimics the upper respiratory tract, but replication is restricted at 37⁰C (Fig. 2C), which the mimics the lower respiratory tract. This property in the DelNS1 flu B virus is an additional safety feature to be used as a vaccine in humans (Figure 2C).

IV. METHODS OF USE

The disclosed AM/LAVIB/DelNS1 can be used to effectively increase viral titer or elicit an immune response in a subject in need thereof. In some aspects, subjects can include the elderly (e.g., >65 years old), young children (e.g., <5 years old). Methods for improving immune response in children using adjuvanted formulations are disclosed for example in U.S. Publication 2017/0202955.

The LAIVB can generally be administered directly to a mammal in need thereof to increase viral titer in the mammal and elicit an immune response. The Examples demonstrate that immunization of one lineage of flu B vaccine, Victoria lineage, is able to protect mice from lethal challenge of either Victoria or Yamagata lineage of mouse adapted flu B virus (Figure 3A and 3B).

Viruses are typically administered to a patient in need thereof in a pharmaceutical composition. Pharmaceutical compositions containing virus may be for systemic or local administration. Dosage forms for administration by parenteral (intramuscular (IM), intraperitoneal (IP), intravenous (IV) or subcutaneous injection (SC)), or transmucosal (nasal, vaginal, pulmonary, or rectal) routes of administration can be formulated. In the most preferred embodiments, the immunizing virus is delivered peripherally by intranasally or by intramuscular injection, and the therapeutic virus is delivered by local injection.

Direct delivery can be accomplished by parenteral injection (e.g., subcutaneously, intraperitoneally, intradermal, intravenously, intramuscularly, or to the interstitial space of a tissue), or mucosally, such as by rectal, oral (e.g., tablet, spray), vaginal, topical, transdermal (See e.g., WO99/27961) or transcutaneous (See e.g., WO02/074244 and WO02/064162), inhalation, intranasal (See e.g., WO03/028760), ocular, aural,

pulmonary or other mucosal administration. Compositions can also be administered topically by direct transfer to the surface of the skin. Topical administration can be accomplished without utilizing any devices, or by contacting naked skin with the composition utilizing a bandage or a bandage-like device (see, e.g., U.S. Pat. No. 6,348,450). In some aspects, the mode of administration is parenteral, mucosal, or a combination of mucosal and parenteral immunizations. In other aspects, the mode of administration is parenteral, mucosal, or a combination of mucosal and parenteral immunizations in a total of 1-2 vaccinations 1-3 weeks apart. In related aspects, the route of administration includes but is not limited to intranasal delivery.

1. Effective amounts

Typically the composition is administered in an effective amount to induce an immune response against a one or more antigens encoded by the virus. For example, an effective amount of virus generally results in production of antibody and/or activated T cells that kill or limit proliferation of or infection by the virus

The composition can typically be used to elicit systemic and/or mucosal immunity, for example to elicit an enhanced systemic and/or mucosal immunity. For example, the immune response can be characterized by the induction of a serum IgG and/or intestinal IgA immune response. Typically, the level of protection against influenza infection can be more than 50%, e.g., 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more. In one aspect, the level of protection can be 100%.

The immune response induced by the invention can be one or both of a TH1 immune response and a TH2 response. The immune response can be an improved or an enhanced or an altered immune response. The immune response can be one or both of a systemic and a mucosal immune response. For example, the immune response can be an enhanced systemic and/or mucosal response. An enhanced systemic and/or mucosal immunity is reflected in an enhanced TH1 and/or TH2 immune response. For example, the enhanced immune response can include an increase in the production of IgG1 and/or IgG2a and/or IgA. In another aspect the mucosal immune response can be a TH2 immune response. For example, the mucosal immune response can include an increase in the production of IgA.

Typically, activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells can typically secrete

one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response can also result in the production of IgG1, IgE, IgA, and/or memory B cells for future protection. In general, a TH2 immune response can include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells. For example, an enhanced TH2 immune response can include an increase in IgG1 production. A TH1 immune response can include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN-gamma, and TNF-alpha), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. For example, the enhanced TH1 immune response can include an increase in IgG2a production.

The LAIVB strains can be used either alone or in combination with other agents optionally with an immunoregulatory agent capable of eliciting a Th1 and/or Th2 response.

2. Dosages

The precise dosage will vary according to a variety of factors such as subject-dependent variables (e.g., age, immune system health, etc.), and age of the subject being treated. Appropriate dosages can be determined by a person skilled in the art, considering the therapeutic context, age, and general health of the recipient. The selected dosage depends upon the desired therapeutic effect, on the route of administration, and on the duration of the treatment desired. In determining the effective amount of the virus to be administered for the prophylaxis, the physician may evaluate circulating plasma levels of virus, and/or the production of existing antibodies against the antigen(s). Active virus can also be measured in terms of plaque-forming units (PFU). A plaque-forming unit can be defined as areas of cell lysis (CPE) in monolayer cell culture, under overlay conditions, initiated by infection with a single virus particle. Generally, dosage levels of virus between 10^2 and 10^{12} pfu are administered to humans. In different embodiments, the dosage range is from 10^4 to 10^{10} pfu, 10^5 to 10^9 pfu, 10^6 to 10^8 pfu, or any dose within these stated ranges. When more than one vaccine is to be administered (i.e., in combination vaccines), the amount of each vaccine agent can be within their described ranges.

Virus is typically administered in a liquid suspension, in a volume ranging between 10 μ l and 100 μ l depending on the route of administration. Vaccine volumes commonly practiced range from 0.1 mL to 0.5 mL. Generally, dosage and volume will be lower for local injection as compared to systemic administration or infusion.

5 The vaccine composition can be administered in a single dose or a multi-dose format. Vaccines can be prepared with adjuvant hours or days prior to administrations, subject to identification of stabilizing buffer(s) and suitable adjuvant composition. Typically, the dose will be 100 μ l administered locally in multiple doses, while systemic or regional administration via subcutaneous, intramuscular, intra-organ, intravenous or
10 intranasal administration can be from for example, 10 to 100 μ l.

V. KITS

A kit including the disclosed LAIVB is also provided. The kit can include a separate container containing a suitable carrier, diluent or excipient. Additionally, the kit can include instructions for mixing or combining ingredients and/or administration.

15 Compositions can be in liquid form or can be lyophilized. Suitable containers for the compositions include, for example, bottles, vials, syringes, and test tubes. Containers can be formed from a variety of materials, including glass or plastic. A container can have a sterile access port (for example, the container can be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle).

20 The kit can further include a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution, or dextrose solution. It can also contain other materials useful to the end-user, including other pharmaceutically acceptable formulating solutions such as buffers, diluents, filters, needles, and syringes or other delivery device(s). The kit can further include a third
25 component comprising an adjuvant.

The kit can also include a package insert containing written instructions for methods of inducing immunity, preventing infections, or for treating infections. The package insert can be an unapproved draft package insert or can be a package insert approved by the Food and Drug Administration (FDA) or other regulatory body.

30 The invention also provides a delivery device pre-filled with the compositions of the invention.

The compositions are generally formulated as sterile, substantially isotonic and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

5

Examples

Materials and Methods

Cells and viruses

B/HK/8038/11 strain was obtained from Queen Mary Hospital and whole genome sequences of DelNS1 B/HK/8038/11 have been deposited in GenBank under accession no:MH636851-MH636858. All cell lines were obtained from ATCC. Human embryonic kidney cells (293T) and lung adenocarcinoma cells (A549) were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate (Life Technologies). Canine MDCK cells were cultured in Eagle's minimal essential medium (MEM) supplemented with the same amount of serum and antibiotics. Influenza B virus B/HK/8038/11 was rescued by reverse genetics. B DelNS1(Flu B) LAIVs were constructed and rescued according to the protocols described here and in the previous report (Zheng, et al, *J. Virol.*, 89:10273-10285 (2015)). Viral gene segments were amplified and cloned into pHW2000 plasmids, resulting in eight pHW2000 plasmids, which were transfected into 293T/MDCK cell mixtures. Rescued virus was amplified in MDCK cells or embryonated chicken eggs.

Construction of plasmids

The NS1 deletion plasmid, pHW2000-DelNS1, was constructed as described before (Zheng, et al, *J. Virol.*, 89:10273-10285 (2015)). Inverse PCR was performed to delete the NS1 gene using pHW2000-8038-NS (influenza B) plasmid as a template. The PCR product was then gel purified, phosphorylated and self-ligated using a standard protocol. Primers for influenza B DelNS1 424 inverse PCR were 5'-CTCAAT TT GTGTTGTGGTC ATG -3' (SEQ ID NO: 25) (Flub-DelNS1-F) and 5'-TGGAGGATGAAGAAGA TGGCCATCGG ATCCTC -3' (SEQ ID NO: 26) (Flub-DelNS1-R).

Example 1. Generation and characterization of DelNS1 influenza B live attenuated virus vaccine.

A clinical isolate, B/Hong Kong/8038/2011, which was confirmed to be the Victoria lineage by sequence analysis, was used in these experiments. Coding region of the NS1 gene was removed from viral genome without affecting the expression of other essential genes for virus replication (**Figure 1A**).

Eight pHW2000 plasmids, containing the DelNS1 segment and the other 7 influenza virus segments, together with an NS1 expression plasmid, were transfected into a 293T/MDCK cell mixture and incubated overnight. The DNA mixture was removed and MEM supplemented with 1 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) added. Virus supernatant was collected 72 h later and designated passage 0 (P0) virus, and then subsequently passaged in MDCK cells or embryonated chicken eggs.

Initial rescued DelNS1-8308B influenza B virus, obtained from transfected 293T cells at 37°C for 72 hours, only replicates poorly in MDCK cells or eggs. Rescued virus was passaged 10 times at 33°C in MDCK cells. For all DelNS1 viruses, deletion of the NS1 gene was confirmed by RT-PCR and sequencing. Rescued virus (DelNS1-8308B influenza B virus) was in MDCK cells for more than 20 times at 33°C and 30°C respectively until virus titer was stabilized, measured as when the virus titer remains unchanged for three consecutive passages. This was followed by identification of adaptive mutations in growth adapted DelNS1-8308B influenza B virus. Four mutations in PA(T210C), NA T(1424C), NP(C182T) and M (A281G) were identified. The DelNS1-8308B influenza B virus, designated Influenza B virus DeINS1-B8038HK, has been deposited with the American Type Culture Collection, and has ATCC Deposit No. PTA-125209.

Example 2. Growth properties of DelNS1 flu B virus in MDCK and A459 cells

Growth kinetics of DelNS1 virus were estimated in MDCK and A549 cells at 37°C and 33°C degrees respectively. Confluent cells (A549 or MDCK) seeded in 24-well plates were infected with viruses at the indicated multiplicity of infection (MOI). After 1 h adsorption, viral supernatant was removed, the cells washed twice with phosphate-buffered saline (PBS), then overlaid with MEM containing 1 µg/ml TPCK trypsin and incubated at the indicated temperature. Supernatants were collected at different time points and titered by plaque assay in MDCK cells.

Plaque assay: Viruses were 10-fold serially diluted and added to confluent MDCK cells in 6-well plates, then incubated at 37°C for 1 h. Supernatant was removed and cells washed twice with PBS and then overlaid with 1% MEM agarose containing 1 µg/ml TPCK trypsin. Plates were incubated at 33°C for 48 h and then fixed with 10% PBS buffered formaldehyde solution for at least 2 h. Plaques were visualized by staining with 1% crystal violet solution.

DelNS1 flu B live attenuated virus with adaptive mutations is able to replicate to comparable level as wild type virus in vaccine producing systems, eggs or MDCK cells (Figure 2A). The DelNS1 live attenuated is not able to replicate in interferon-competent cells, A549. This a safety property to prevent virus shedding in vaccinated humans (Figure 2B).

Growth kinetics of DelNS1-8308B influenza B virus carrying adaptive mutations was analyzed at 33°C and 37°C in MDCK cells. The DelNS1 flu B virus prefer to replicate at lower temperature, such as 33°C, which is mimics the upper respiratory tract, but replication is restricted at 37°C, which is the mimics the lower respiratory tract. This property in the DelNS1 flu B virus is an additional safety feature to be used as a vaccine in humans (Figure 2B).

Example 3. Protection of lethal challenge by mouse adapted flu B viruses with DelNS1 flu B vaccine

Mice were vaccinated with DelNS1 Flu B vaccine (Victoria lineage, 2×10^6 pfu) ONCE through nasal drop.

After three weeks, vaccinated mice were subsequently challenged with either one of mouse adapted flu B viruses, B/Florida/4/2006 (Yamagata lineage, 5×10^5 TCID₅₀)– and B/Brisbane/60/ 2008 (Victoria lineage, 5×10^6 TCID₅₀). Body weight and survival rate of the mice were recorded for 2 weeks. Mice with body weight loss greater than 25% were euthanized, in accordance with animal ethics guidelines. To evaluate viral replication in mouse lungs, lungs were collected at day 3 and homogenized in 1 ml PBS. Viral titers were determined by plaque assay in MDCK cells.

DelNS1 flu B virus is not virulent to mice, even when challenged with the highest dose of virus (Figure 3A and 3B). To assess if DelNS1-B8038, a Victoria lineage influenza B virus, protects from infection with both lineages of influenza B virus, mice were immunized once through the nasal route, then after three weeks challenged with

mouse-adapted influenza B virus of either the Yamagata (B/Florida/4/2006) or Victoria (B/Brisbane/60/2008) lineage. Mice mock vaccinated with PBS died after 6-8 days, whereas all DelNS1-B8038 LAIV immunized mice survived (Figure 3C and 3D). Virus titers in lungs at day three post infection were significantly lower in DelNS1-B8038

5 LAIV vaccinated mice compared to the control group (Fig. 4A and B). Immunization of one lineage of flu B live attenuated DelNS1 vaccine, DelNS1-B8038 of Victoria lineage, is able to protect mice from lethal challenge of either Victoria or Yamagata lineage of mouse adapted flu B virus (Figure 3A-3D).

10 It is understood that the disclosed method and compositions are not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

15 It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "an aptamer" includes a plurality of such aptamers, reference to "the aptamer" is a reference to one or more aptamers and equivalents thereof known to those skilled in the art, and so forth.

20 Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps.

25 "Optional" or "optionally" means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

30 Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, also specifically contemplated and considered disclosed is the range from the one particular value and/or to the other particular value unless the context specifically indicates otherwise. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another, specifically contemplated embodiment that should be considered disclosed unless the context specifically indicates

otherwise. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint unless the context specifically indicates otherwise. It should be understood that all of the individual values and sub-ranges of values contained within an explicitly disclosed range are also specifically contemplated and should be considered disclosed unless the context specifically indicates otherwise. Finally, it should be understood that all ranges refer both to the recited range as a range and as a collection of individual numbers from and including the first endpoint to and including the second endpoint. In the latter case, it should be understood that any of the individual numbers can be selected as one form of the quantity, value, or feature to which the range refers. In this way, a range describes a set of numbers or values from and including the first endpoint to and including the second endpoint from which a single member of the set (i.e. a single number) can be selected as the quantity, value, or feature to which the range refers. The foregoing applies regardless of whether in particular cases some or all of these embodiments are explicitly disclosed.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed method and compositions belong. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present method and compositions, the particularly useful methods, devices, and materials are as described. Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such disclosure by virtue of prior invention. No admission is made that any reference constitutes prior art. The discussion of references states what their authors assert, and applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of publications are referred to herein, such reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

Although the description of materials, compositions, components, steps, techniques, etc. may include numerous options and alternatives, this should not be construed as, and is not an admission that, such options and alternatives are equivalent to each other or, in particular, are obvious alternatives. Thus, for example, a list of different

moieties does not indicate that the listed moieties are obvious one to the other, nor is it an admission of equivalence or obviousness.

CLAIMS

We claim:

1. A live attenuated influenza virus (LAIV) B “LAIVB”) comprising a deletion in the coding region for NS1 (nonstructural protein 1), LAVIB/DelNS1 and one or more mutations selected from the group consisting of PA(T210C), NA T(1424C), NP(C182T) and M (A281G) (AM/ LAVIB/DelNS1).
2. The attenuated virus of claim 1, wherein the virus is not able to replicate in interferon-competent cells
3. The virus of claim 2, wherein the interferon-competent cells are A549 cells.
4. The virus of any one of claims 1-3, wherein the virus replicates poorly in MDCK cells at 37 °C, when compared to its replication at 33 °C in the MDCK cells.
5. The virus of any one of claims 1-4, wherein the virus is able to replicate at levels comparable to wild type influenza virus of the same strain, in a vaccine producing system, optionally, wherein between 10⁷ pfu/ml and 10⁸ pfu/ml of AM/ LAVIB/DelNS1 is recovered from the supernatant following passage in a virus producing system.
6. The virus of claim 6 wherein the vaccine producing system comprises MDCK cells culture.
7. The virus of any one of claims 1-6, wherein administration of the virus to a subject in need thereof reduces viral titer of influenza B virus of a different strain in the subject infected with a different strain of influenza B virus.
8. A method for making LAIVB with a deletion of the viral virulence element, the NS1 protein (DelNS1) and an adaptive mutations which allows growth of the mutated strain in vaccine producing systems such as eggs and MDCK cells comprising (a) generating influenza B virus with deletion of the coding region of the NS1 coding region by transfecting the DelNS1 influenza B virus into one or more vaccine producing cells (b) rescuing LAVIB/DelNS1 and (c) passaging rescued virus culturing into one or more vaccine producing cells until viral titer is stabilized, to obtain AM/ LAVIB/DelNS1.
9. The method of claim 8, wherein the genome of LAIVB/DelNS1 is transfected into a 293T/MDCK cell mixture.
10. The method of claim 9, further comprising passaging rescued virus in MDCK cells until virus titer is stabilized, wherein stabilized viral titre is measured as virus titer remaining unchanged for at least three consecutive passages in MDCK cells or embryonated chicken eggs.

11. The method of any one of claims 8-10 wherein the influenza B virus is a B/Yamagata or B/Victoria strain.
12. The method of claim 11, wherein the mutations following step (b) are PA(T210C), NA T(1424C), NP(C182T) and M (A281G) mutations.
13. The method of claim 12, wherein the virus is Victoria strain.
14. A LAIVB attenuated virus made according to the methods of claims 8-13.
15. A pharmaceutical composition comprising an effective amount of a virus to induce an immune response to an antigen encoded by virus's genome, the virus's genome comprising a deletion in the NS1 protein coding region and one or more mutations following transfection of a DelNS1 influenza B virus into one or more vaccine producing cells and passaging rescued virus culturing into one or more vaccine producing cells until viral titer is stabilized.
16. A pharmaceutical composition comprising an effective amount of a virus to induce an immune response to an antigen encoded by virus's genome, the virus's genome comprising a deletion in the NS1 protein coding region and PA(T210C), NA T(1424C), NP(C182T) and M (A281G) mutations.
17. The composition of claim 15 or 16, further comprising an adjuvant.
18. The composition of any one of claims 15-17, suitable for nasal administration.
19. A method for increasing an immune response in a subject in need thereof, comprising administering the composition of any one of claims 15-18, to the subject.

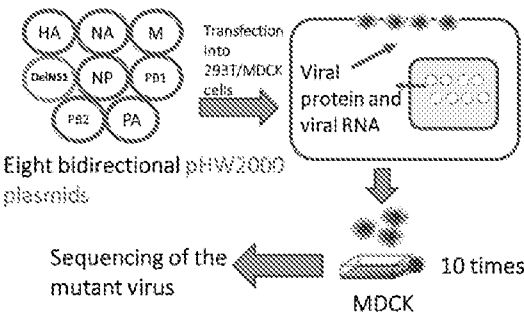


FIG. 1A

Segment	Mutations
PA	T210C
NA	T1424C
NP	C182T
M	A281G

FIG. 1B

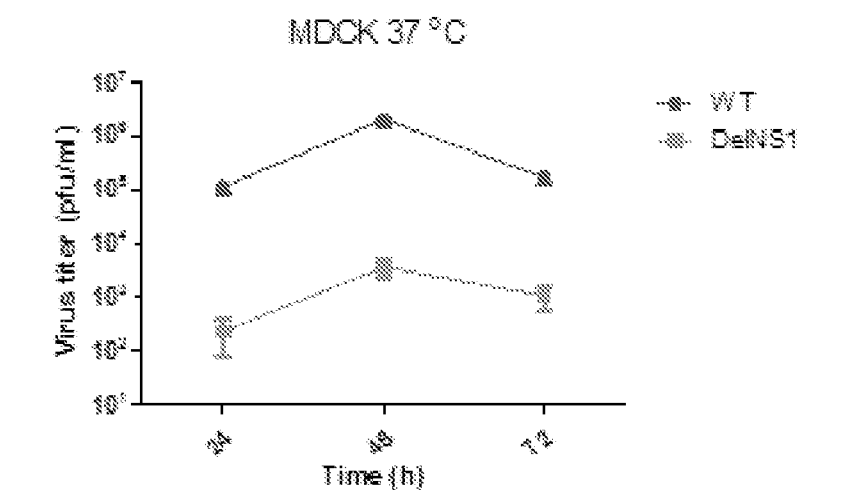


FIG. 2A

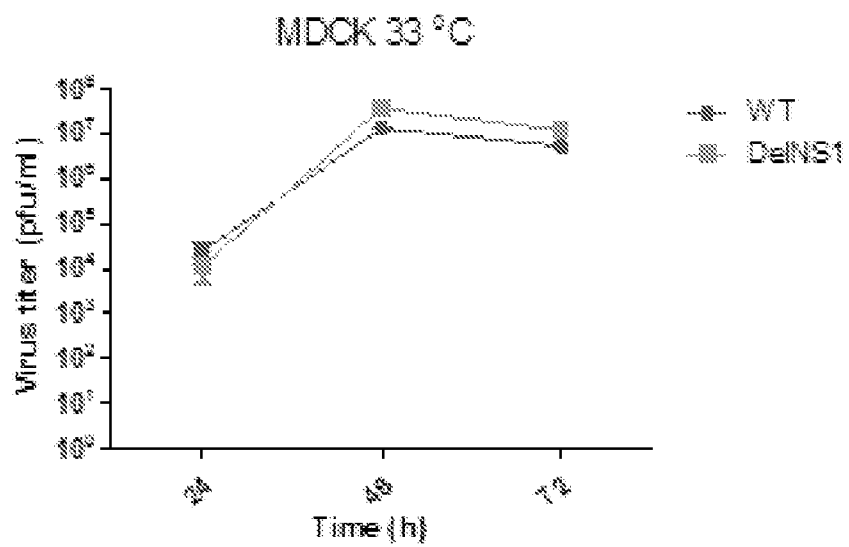


FIG. 2B

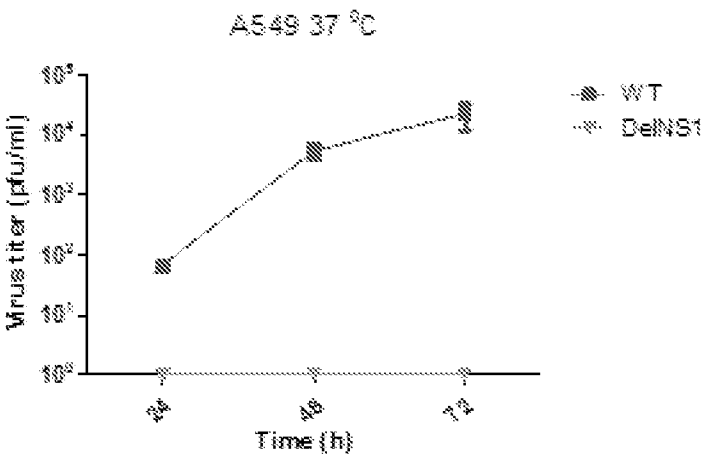


FIG. 2C

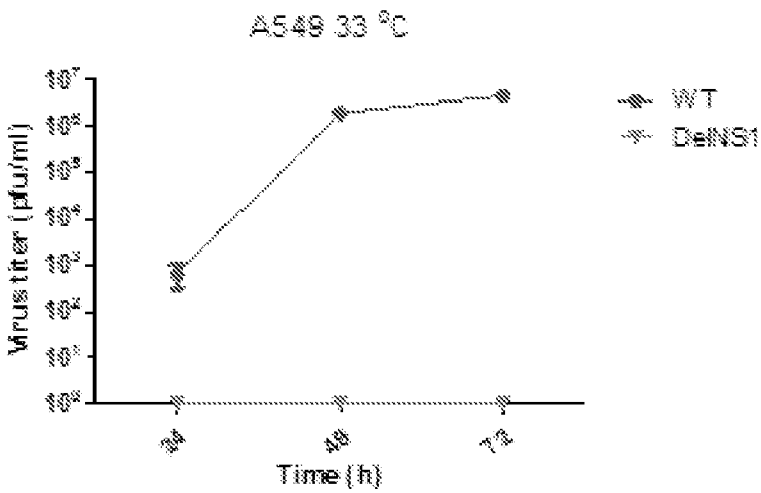


FIG. 2D

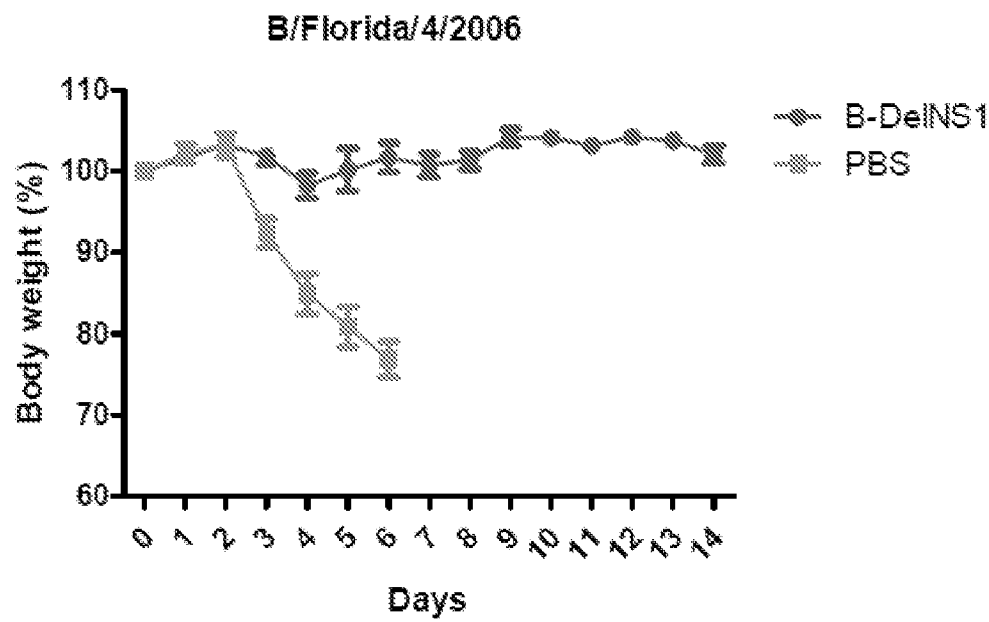


FIG. 3A

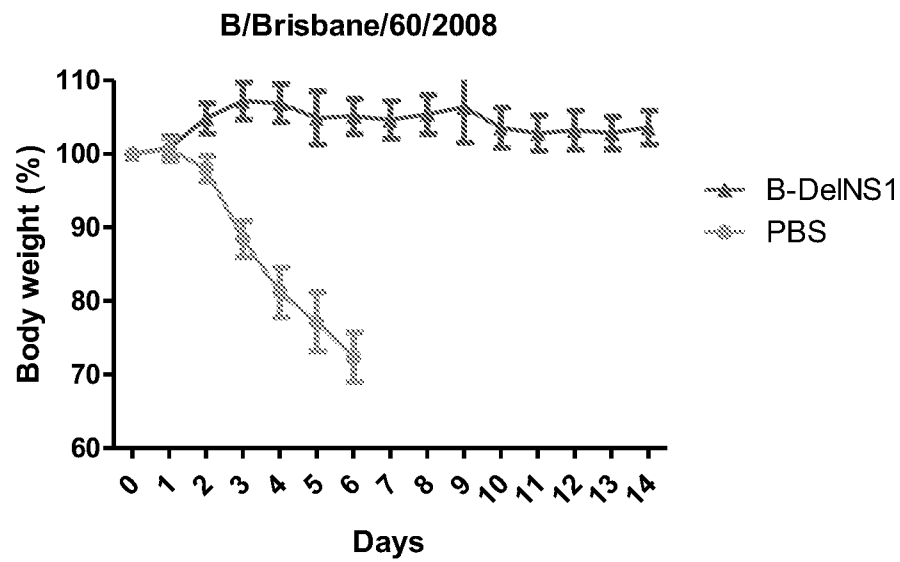


FIG. 3B

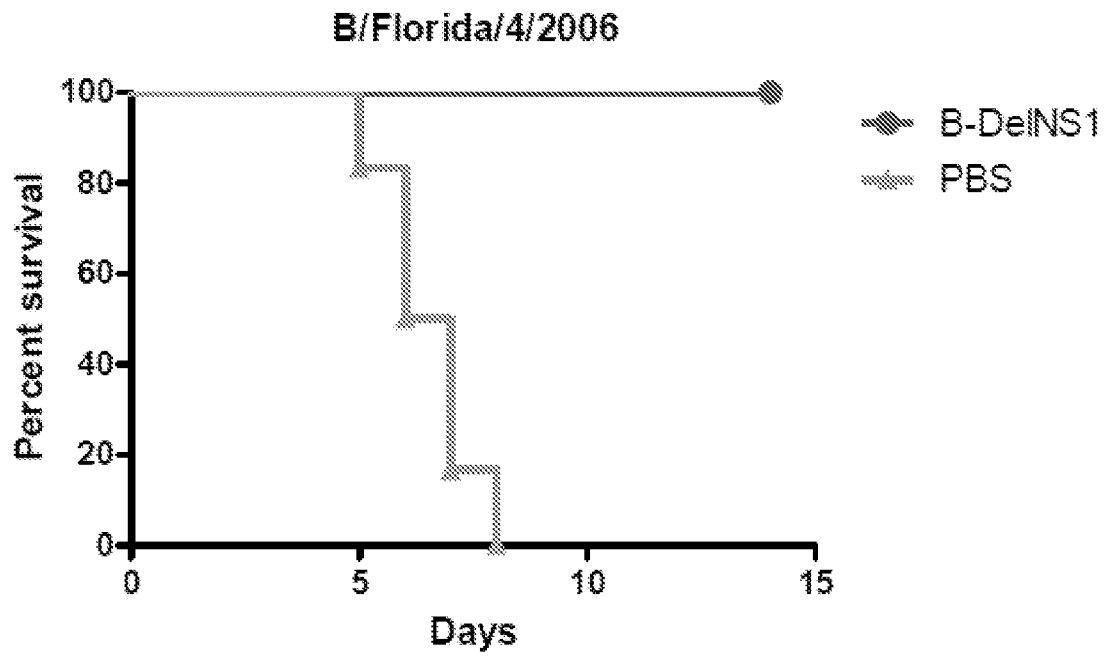


FIG. 3C

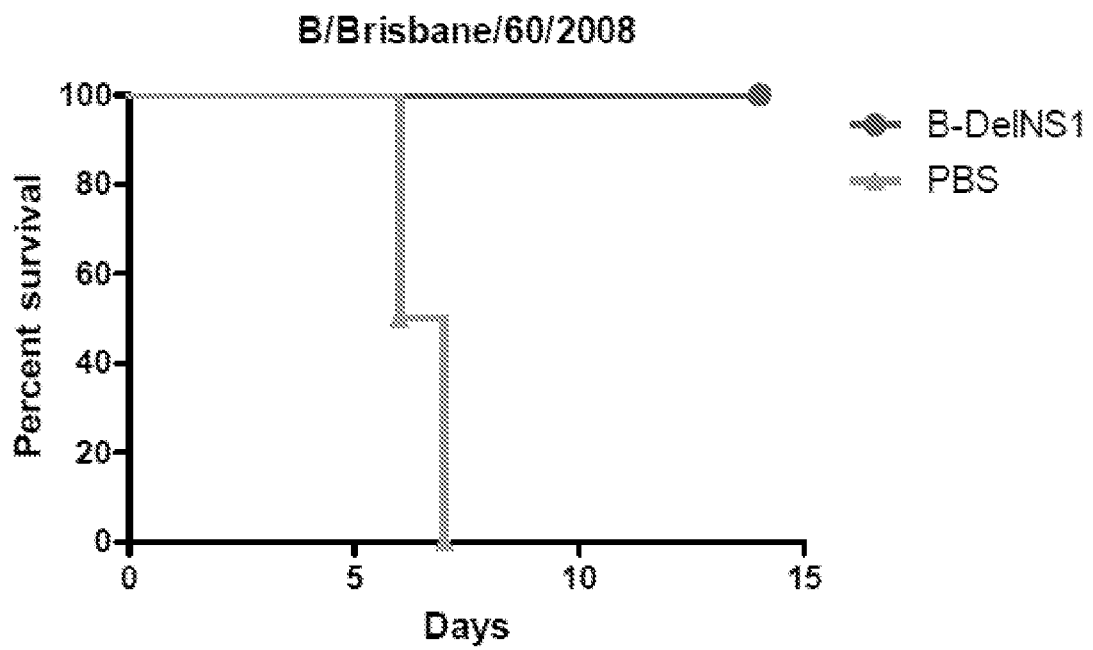


FIG. 3D

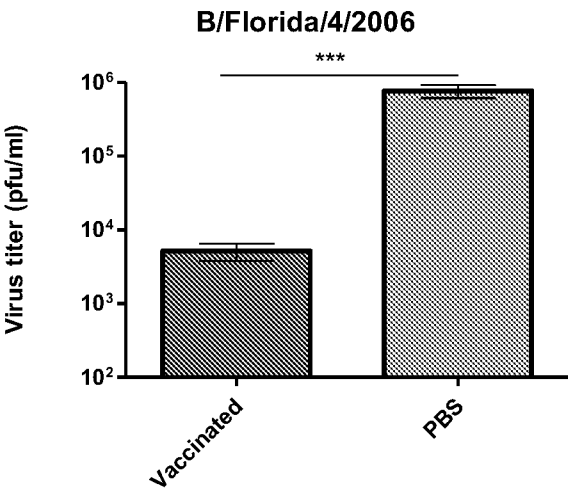


FIG. 4A

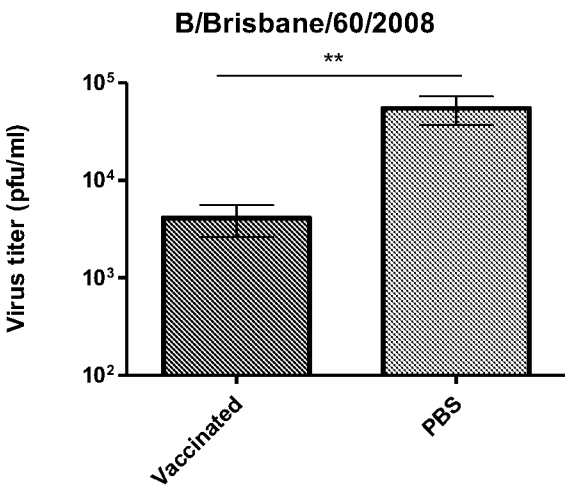


FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/115938

A. CLASSIFICATION OF SUBJECT MATTER

C12N 7/04(2006.01)i; A61K 39/145(2006.01)i; A61P 31/16(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N,A61K,A61P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DWPI, SIPOABS, CNABS,USTXT, JPTXT, EPTXT, WOTXT, CNTXT, cnki, isi web of science:adaptive mutation, influenza virus,b, ns1, deletion,mutation,knockout, c182t, rescue,t210c,1424C,C182T,A281G, PA, NA T, NP, M

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 107208071 A (VERSITECH LTD) 26 September 2017 (2017-09-26) claims 1-24, description ,the paragraphs[0040][0049],examples 1-9	8-11, 14, 15, 17-18
A	CN 107208071 A (VERSITECH LTD) 26 September 2017 (2017-09-26) claims 1-24, description ,the paragraphs[0040][0049],examples 1-9	1-7, 12,13, 16
A	CN 101796190 A (AVIR GREEN HILLS BIOTECHNOLOGY) 04 August 2010 (2010-08-04) see the whole document	1-18
A	US 2009232843 A1 (NIMAN HENRY L) 17 September 2009 (2009-09-17) see the whole document	1-18
A	Nina Wressnigg等. "Development of a live-attenuated influenza ANS1 intranasal vaccine candidate" <i>Vaccine</i> , Vol. 27, 11 March 2009 (2009-03-11), ISSN: 0264-410x, pages 2851-2857 see the whole document	1-18

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

12 July 2019

Date of mailing of the international search report

08 August 2019

Name and mailing address of the ISA/CN

National Intellectual Property Administration, PRC
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100088
China

Authorized officer

WANG,Qiyang

Facsimile No. (86-10)62019451

Telephone No. 62411211

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/115938

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2018/115938

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **19**
because they relate to subject matter not required to be searched by this Authority, namely:
[1] Methods for treatment of the human or animal body by surgery or therapy.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2018/115938

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	107208071	A	26 September 2017	EP	3218470	A4	11 April 2018
				EP	3218470	A1	20 September 2017
				AU	2015345579	B2	21 February 2019
				WO	2016074644	A1	19 May 2016
				US	9827304	B2	28 November 2017
				AU	2015345579	A1	04 May 2017
				US	2016136261	A1	19 May 2016