

MODIFIED ALPHAVIRAL nsP3**FIELD**

The present disclosure relates to modified alphaviral non-structural protein 3 (nsP3) and self-amplifying RNA (saRNA) encoding the same, in particular wherein the saRNA is derived from alphaviruses and the sequence encoding nsP3 is modified to increase safety while maintaining gene expression capacity. The disclosure further relates to methods of producing such engineered saRNA, and the use thereof in medicine, such as in vaccination.

BACKGROUND

Self-replicating RNA, or "replicon" RNA, is used as a vector for transgene expression in vitro and in vivo. Alphavirus-based replicons derived from many different species of alphavirus (e.g., Venezuelan equine encephalitis virus (VEEV), Sindbis virus, Semliki Forest virus) have been used as vectors for transgene expression both in vitro and in vivo. These vectors rely on the activity of non-structural viral proteins encoded therein to replicate the replicon and to mediate expression of the protein(s) encoded in the place of the structural gene open reading frame (ORF) that is translated from sub genomic (SG) mRNAs synthesized late in the replicative life cycle.

Self-amplifying RNA, even more so than conventional mRNA, activate the innate immune system. Such innate immune response can both support or hamper the therapeutic or prophylactic use of saRNA constructs. Strategies are reported to reduce the innate immune-stimulating activity of RNA, some of which may also be applicable to saRNA. However, strategies to reduce the innate immune sensing of conventional mRNA, are not necessarily believed to be well tolerated in functional saRNA (Minnaert et al. Adv Drug Delivery Rev. 176 (2021) 113900).

Few reports are available in literature referring to replicon RNA modified in the non-structural proteins, in particular encoding modified nsP3. US 2006/0251678 describes VEEV replicon RNA encoding a mutated non-structural protein nsP3 having a Pro residue at amino acid 121. Li Y. et al. (Nature Scientific Reports (2019) 9:6932) reports enhanced RNA replicons for immunotherapy and reports six mutations in the VEEV non-structural proteins, two of which in nsP2 and four in nsP3. US2020/0109178 provides replicon RNA encoding for a hybrid nsP3 combining nsP3 fragments derived from Old World and New World alphaviruses in the hypervariable domain of nsP3, in an attempt to diminish or eliminate the immune response against the heterologous protein encoded by the replicon.

BRIEF SUMMARY

Several modified forms of alphaviral replicon RNAs have been reported providing for different effects on the functionality of the replicon RNA. However, there remains a need for saRNA vectors tailored to reduce the innate immune response whilst being proficient in delivering the payload encoded by a heterologous gene of interest comprised in the replicon RNA. The present disclosure provides modified forms of alphaviral non-structural protein 3 (nsP3) sequences which confer enhanced functionality of the overall replicon RNA construct, such as in an alphavirus replicon RNA.

Accordingly, in a first aspect, an alphaviral non-structural protein 3 (nsP3) is provided which is modified at least by a mutation at amino acid position equivalent to amino acid position 167 of wild-type Venezuelan equine encephalitis virus (VEEV) nsP3 of SEQ ID NO:1.

In some embodiments, the alphaviral nsP3 comprises a Met residue at amino acid position equivalent to amino acid position 167 of wild-type VEEV nsP3.

In some embodiments, the alphaviral nsP3 is a VEEV nsP3 modified at least by a mutation at amino acid position 167, optionally a I167M mutation. In some embodiments, the VEEV nsP3 has an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:18.

In a further aspect, a nucleic acid is provided comprising a sequence encoding an alphaviral nsP3 described herein. In some embodiments, the sequence encoding the nsP3 has a nucleotide sequence of SEQ ID NO: 8, or at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical in sequence to SEQ ID NO: 8 provided the sequence comprises the A501G mutation of SEQ ID NO: 8.

In a further aspect, a replicon RNA is provided comprising a sequence encoding alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, wherein said alphaviral nsP3 is an alphaviral nsP3 comprising at least a single amino acid mutation as described herein.

In a further aspect, a replicon RNA is provided comprising a sequence encoding alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, wherein said sequence is modified at least by a modification at the nucleotide position equivalent to position 4488 of the sequence encoding wild-type VEEV nsPs 1-4 of SEQ ID NO:3.

The alphavirus from which the sequence encoding the non-structural proteins are derived may be selected from VEEV (such as TC-83 VEEV or Trinidad donkey virus), Everglades virus, Tonate virus, Mucambo virus Cabassou virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus and variants thereof. Optionally, the sequence
5 encoding the alphaviral non-structural proteins are derived from VEEV.

In some embodiments, the sequence encoding the alphaviral nsPs1-4 is derived from VEEV, or has at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the wild-type sequence encoding VEEV nsPs1-4 of SEQ ID NO:3.

10 In some embodiments, the sequence encoding the alphaviral nsPs1-4 has a nucleotide sequence of SEQ ID NO:4.

In some embodiments, the replicon RNA further comprises a 5'UTR, one or more heterologous sequences encoding one or more heterologous gene products, wherein each heterologous sequence is operably linked to one or more subgenomic promoters
15 and/or IRES elements, and a 3'UTR.

In some embodiments, one or more of the heterologous sequences encodes a heterologous protein. The heterologous protein may be an antigenic protein. The heterologous protein may be a therapeutic protein.

In a further aspect, a method is provided for expressing one or more heterologous
20 gene products in a cell, comprising: introducing a replicon RNA as described herein into a cell; and expressing the one or more heterologous gene products encoded by said replicon in the cell.

In a further aspect, a cell is provided comprising a replicon RNA described herein, optionally wherein the cell is a mammalian cell, such as a human cell.

25 In a further aspect, a nanoparticle is provided comprising a replicon RNA described herein, optionally wherein the nanoparticle is a lipid nanoparticle.

In a further aspect, a pharmaceutical composition is provided comprising a replicon RNA described herein, a cell described herein, or a nanoparticle described herein.

In a further aspect, a replicon RNA described herein, a cell described herein, a
30 nanoparticle described herein, or a pharmaceutical composition described herein is provided for use in medicine, optionally for use in a method of inducing an (adaptive) immune response in an individual, such as a method of vaccination.

In a further aspect, a method of treating a disease or condition in a human subject in need thereof, comprising: administering a pharmaceutical composition comprising

a replicon RNA according to any of the exemplary aspects/embodiments described herein to the human subject, thereby causing expression of the replicon RNA in one or more cells, tissues, or organs of the human subject. In some embodiments, the replicon RNA is administered in a therapeutically effective amount. In some
5 embodiments, the therapeutically effective amount comprises an amount sufficient to cause expression of an amount of a heterologous protein that elicits an adaptive immune response.

In a further aspect, a DNA template encoding a replicon RNA comprising the nucleic acid encoding the nsP3 described herein is provided.

10 In a further aspect, a method for making the replicon RNA comprising the nucleic acid encoding the nsP3 described herein is provided.

DESCRIPTION OF THE FIGURES

Figure 1 - In vitro luciferase expression in Hela cells: luciferase expression (panels A & B) and cell viability (panels C & D) in Hela cells either mock transfected
15 or transfected with construct 1 (parental wild-type VEEV saRNA), construct 2 (saRNA encoding VEEV nsP3 with I167M mutation) or conventional mRNA, each encoding luciferase. Expression reported at 24 hours post transfection (hpt) (panels A and C) or 48 hpt (panels B and D).

Figure 2 - In vitro luciferase expression in RD cells: luciferase expression (panel
20 A) and cell viability (panel B) in RD cells either mock transfected or transfected with construct 1 (parental wild-type VEEV saRNA), construct 2 (saRNA encoding VEEV nsP3 with I167M mutation) or conventional mRNA encoding luciferase at 24 hpt.

Figure 3 - Protein expression kinetics in Hela cells: mean fluorescence intensity (MFI) of all cells either mock transfected or after transfection with construct 3
25 (parental wild-type VEEV saRNA), construct 4 (saRNA encoding VEEV nsP3 with I167M mutation) or conventional mRNA encoding eBFP.

Figure 4 - Expression of subgenomic RNA in Hela cells: normalized counts of the total subgenomic RNA in Hela cells either mock transfected or after transfection with parental saRNA (wild-type VEEV nsPs) or saRNA encoding VEEV nsP3 with the
30 I167 mutation.

Figure 5 - Volcano plot of transcripts of the IFN-alpha pathway: differential expression analysis of cells transfected with saRNA encoding VEEV nsP3 with the I167 mutation compared to the parental saRNA (wild-type VEEV nsPs): transcripts that are part of the IFN-alpha pathway are shown in black, all other transcripts are shown
35 in grey; dots on the left half of the graph (left of point 0 in the X-axis) represent

transcripts that are less abundant in cells transfected with saRNA encoding VEEV nsP3 with the I167 mutation compared to the parental saRNA whereas dots on the right half of the graph (right of point 0 in the X-axis) represent transcripts that are more abundant in cells transfected with saRNA encoding VEEV nsP3 with the I167 mutation compared to the parental saRNA.

Figure 6 - Protein expression kinetics in vivo: total flux of luciferase activity in mice that were administered an empty LNP (mock) or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M) or conventional mRNA (mRNA) encoding luciferase measured over time.

Figure 7 - Serum IgG titers – Anti-HA IgG concentrations in serum are reported as determined 50 days after first injection (29 days after second injection) with an empty LNP or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M) or conventional mRNA (mRNA) encoding hemagglutinin (HA).

Figure 8 - Cytokine expressing CD8+ T-cells – Percentage of cytokine expressing CD8+ T-cells on the total of CD8+ T-cells after HA peptide stimulation are reported 50 days after first injection (29 days after second injection) with an empty LNP (mock) or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M) or conventional mRNA (mRNA) encoding HA; single, double and triple positives refers to the number of CD8+ T-cells expressing respectively one, two or all three of IL2, TNF-alpha or IFN-gamma.

Figure 9 - Cytokine expressing CD4+ T-cells – Percentage of cytokine expressing CD4+ T-cells on the total number of CD4+ T-cells after HA peptide stimulation were determined 50 days after first injection (29 days after second injection) with an empty LNP (mock) or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M) or conventional mRNA (mRNA) encoding HA; single, double and triple positives refers to the number of CD4+ T-cells expressing respectively one, two or all three of IL2, TNF-alpha or IFN-gamma.

Figure 10: Serum cytokine levels - Serum cytokine levels for interferon gamma, KC, MCP1, IP10, interferon alfa and IL-6 are reported as measured by multiplex ELISA one day after a first and a second injection (22 days after first injection) with an empty LNP (mock) or an LNP complexed with saRNA comprising wt VEEV nsPs

(parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M) or conventional mRNA (mRNA) encoding HA.

Figure 11: Tissue Cytokine expression in the lymph nodes and site of injection - Transcript levels for IFN α 2 and IFN β 1 in the lymph node and muscle (site of injection) are reported as measured by RT-qPCR one day after a first and a second injection (21 days after first injection) with an empty LNP (mock) or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M) or conventional mRNA (mRNA) encoding HA: results are expressed as log2 fold change relative to empty LNP (Mock).

Figure 12 – non-structural protein based homology alphaviruses – schematic representation of alphavirus variants closest to wild-type VEEV bases on homology in the non-structural proteins.

Figure 13 – Percentage of eBFP expressing human moDC cells – Percentage of cells either mock transfected or after transfection with construct 3 (parental wild-type VEEV saRNA), construct 4 (saRNA encoding VEEV nsP3 with I167M mutation) or conventional mRNA encoding eBFP.

Figure 14 – Level of eBFP expression in human moDC cells: Mean Fluorescence Intensity (MFI) of all cells either mock transfected or after transfection with construct 3 (parental wild-type VEEV saRNA), construct 4 (saRNA encoding VEEV nsP3 with I167M mutation) or conventional mRNA encoding eBFP.

Figure 15 – RT-qPCR of IFNB1: IFNB1 transcript levels as determined by qPCR relative to mock transfected Hela cells of cells transfected with constructs 1 (parental 1), construct 2 (parental 1 with I167M mutation), constructs 7 (parental 2), construct 8 (parental 2 with I167M mutation), constructs 9 (parental 3), construct 10 (parental 3 with I167M mutation).

Figure 16 – RT-qPCR of OAS1: OAS1 transcript levels as determined by qPCR relative to mock transfected Hela cells of cells transfected with constructs 1 (parental 1), construct 2 (parental 1 with I167M mutation), constructs 7 (parental 2), construct 8 (parental 2 with I167M mutation), constructs 9 (parental 3), construct 10 (parental 3 with I167M mutation).

Figure 17 – RT-qPCR of PKR: PKR transcript levels as determined by qPCR relative to mock transfected Hela cells of cells transfected with constructs 1 (parental 1), construct 2 (parental 1 with I167M mutation), constructs 7 (parental 2), construct 8 (parental 2 with I167M mutation), constructs 9 (parental 3), construct 10 (parental 3 with I167M mutation).

Figure 18 - Serum IgG titers – Anti-Spike IgG concentrations in serum are reported as determined 42 days after first injection (14 days after second injection) with an empty LNP or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M), saRNA encoding VEEV nsP3 with the I167M mutation containing m5C (I167M + m5C) or saRNA encoding codon optimized VEEV nsPs with the I167M mutation in nsp3 (Codon optimized I167M) encoding SARS-COV2 Spike protein.

Figure 19 - Cytokine expressing CD8+ T-cells – Percentage of cytokine expressing CD8+ T-cells on the total of CD8+ T-cells after Spike peptide stimulation are reported 42 days after first injection (14 days after second injection) with an empty LNP or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M), saRNA encoding VEEV nsP3 with the I167M mutation containing m5C (I167M + m5C) or saRNA encoding codon optimized VEEV nsPs with the I167M mutation in nsp3 (Codon optimized I167M) encoding SARS-COV2 Spike protein; single, double and triple positives refers to the number of CD8+ T-cells expressing respectively one, two or all three of IL2, TNF-alpha or IFN-gamma.

Figure 20 - Cytokine expressing CD4+ T-cells – Percentage of cytokine expressing CD4+ T-cells on the total of CD4+ T-cells after Spike peptide stimulation were determined 42 days after first injection (14 days after second injection) with an empty LNP or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M), saRNA encoding VEEV nsP3 with the I167M mutation containing m5C (I167M + m5C) or saRNA encoding codon optimized VEEV nsPs with the I167M mutation in nsp3 (Codon optimized I167M) encoding SARS-COV2 Spike protein; single, double and triple positives refers to the number of CD4+ T-cells expressing respectively one, two or all three of IL2, TNF-alpha or IFN-gamma.

Figure 21 - Serum cytokine levels - Serum cytokine levels for interferon gamma, CXCL1, CCL2, CXCL10, interferon alfa and IL-6 are reported as measured by multiplex ELISA one day after a first and a second injection (29 days after first injection) with an empty LNP or an LNP complexed with saRNA comprising wt VEEV nsPs (parental), saRNA encoding VEEV nsP3 with the I167M mutation (I167M), saRNA encoding VEEV nsP3 with the I167M mutation containing m5C (I167M + m5C) or saRNA encoding codon optimized VEEV nsPs with the I167M mutation in nsp3 (Codon optimized I167M) encoding SARS-COV2 Spike protein.

Figure 22 – schematic representation of the schedule of the pig trial of example 11 until day 56.

DETAILED DESCRIPTION

The present disclosure provides replicon RNAs engineered to reduce anti-vector immunogenicity (e.g. innate immunity) without compromising on the expression of an encoded gene of interest.

Surprisingly, the present inventors have identified replicon RNAs, i.e. alphavirus replicon RNA comprising an alphavirus nsP3 modified as described herein, which following transfection in a cell produce a reduced amount RNA (mRNA) whilst retaining the ability to produce similar amounts of the payload compared to the replicon RNA without the modifications provided herein, or, at least sufficient amounts of the payload encoded by the one or more genes of interest in the saRNA to exert its intended function. High RNA content has been identified as a key factor in the reactogenicity of RNA therapeutics, in particular in triggering innate immune responses. However, attenuating mutations in the alphaviral replicon nsP regions leading to reduced RNA expression typically also lead to decreased (peak) expression of the payload/heterologous gene of interest. The examples described herein show that, surprisingly, a replicon RNA encoding an alphavirus nsP3 comprising a mutation as described herein maintains an expression profile (of the GOI/heterologous protein) similar to the replicon encoding the wild-type alphaviral nsP3 from which it is derived, despite generating reduced levels of subgenomic RNA. As further illustrated by the data, the reduced amount of RNA leads to decrease in markers related to reactogenicity of the replicon RNA (such as serum levels of pro-inflammatory cytokines).

At the same time, since the nsP3 modifications disclosed herein do not substantially affect the expression of the GOI/heterologous protein encoded by the replicon, transfection using saRNA encoding an alphavirus nsP3 described herein enables a broad adaptive immune response to an (antigenic) heterologous protein encoded by the saRNA. The induced adaptive immune response includes both humoral and cellular mediated response, and, as demonstrated in the examples, notably a much higher CD8+ response compared to conventional mRNA. This broad adaptive immune response including a humoral and cell mediated immune response is particularly beneficial in a vaccination context, both prophylactic and therapeutic. Alternatively, the maintained levels of GOI expression whilst reducing reactogenicity may also be beneficial in a therapeutic setting where the replicon RNA encodes a therapeutic protein for protein replacement therapy.

Unless defined otherwise, all technical and scientific terms used herein have the meaning as commonly understood to one of ordinary skill in the art. Otherwise, certain terms used herein have the meanings as set forth in the specification.

It must be noted "a", "an", and "the" as used herein refers to both singular and plural referents unless the context clearly dictates otherwise. The term "at least", preceding
5 a series of elements is to be understood to refer to every element in the series. Throughout the specification and the claims, unless the context requires otherwise, the word "comprise" and variations thereof will be understood to imply the inclusion of a stated integer, step or component (or group thereof) but not the exclusion of
10 any other integer, step or component. The term "comprising" and variations thereof may be substituted with the term "containing", "including" or sometimes "having".

When used herein, "consisting of" excludes any integer, element, step or component not specified in the sentence. When used, "consisting essentially of" does not exclude elements, steps or components that do not materially affect the basic and novel
15 characteristics of the subject matter defined by the sentence.

As used herein, the term "nucleotide" or "nucleotides" refers to the nucleotide bases that are the basic building blocks of nucleic acids. A nucleotide consists of a sugar molecule (either ribose in RNA or deoxyribose in DNA) attached to a phosphate group and a nitrogen-containing base (either adenine, cytosine, guanine or uracil in RNA,
20 or, adenine, cytosine, guanine or thymine in DNA). Unless specified otherwise, the nucleotides for defining the replicon RNA constructs or sequences disclosed herein are ribonucleotides. Alternatively, DNA templates used for the manufacture of the replicons disclosed herein are deoxyribonucleotides.

The term "nucleic acids" refers to long chainlike molecules composed of a series of
25 nucleotides including both RNA and DNA molecules. The term "nucleic acid sequence" refers to the sequence of nucleotides conventionally going from the 5'-end to the 3'-end of the linear nucleic acid.

The term "recombinant" or "engineered" nucleic acid or protein as used herein, refers to the fact that the nucleic acid to which it pertains has been altered through human
30 intervention.

When used herein, "replicon RNA", used interchangeably with "replicon", "self-amplifying RNA" ("saRNA") or "self-replicating RNA" ("srRNA") refers to an RNA molecule that, contrary to conventional mRNA, contains all of the genetic information required for directing its own amplification or self-replication within a permissive cell.
35 Typically, replicon RNA constructs are engineered from genomes of plus-strand RNA

viruses. To direct its own amplification, the RNA molecule encodes an enzyme complex for directing its own amplification in vivo in a target cell and contains the necessary elements required for replication and which are recognized and utilized by the encoded polymerases.

5 The term "alphavirus" and its derivations have their conventional meaning in the art and includes various species in Alphavirus genus of the Togoviridae family such as Venezuelan equine encephalitis virus (VEEV; e.g. particular strains referred to as Trinidad donkey, TC-83, etc.), Sindbis virus, Western equine encephalitis virus (WEEV), Eastern Equine Encephalitis Virus (EEEV), Chikungunya virus, Everglades
10 virus, Tonate virus, Mucambo virus, Cabassou virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus, Madriaga virus, Highlands J virus, Fort Morgan virus, and other closely related variants. In some embodiments, the replicon elements are derived from an alphavirus selected from VEEV (e.g. particular strains referred to as Trinidad donkey, TC-83, etc.), Sindbis virus, WEEV, EEEV, Chikungunya virus,
15 Everglades virus, Tonate virus, Mucambo virus Cabassou virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus, Madriaga virus, Highlands J virus, and Fort Morgan virus. In some embodiments, the replicon elements are derived from an alphavirus selected from VEEV (e.g. particular strains referred to as Trinidad donkey, TC-83, etc.), WEEV, EEEV, Everglades virus, Tonate virus, Mucambo virus Cabassou
20 virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus, Madriaga virus, Highlands J virus, Fort Morgan virus. In some embodiments, the replicon elements are derived from an alphavirus selected from VEEV (e.g. particular strains referred to as TC-83, Trinidad donkey etc.), Everglades virus, Tonate virus, Mucambo virus Cabassou virus, Mosso das pedras virus, Rio Negro virus, and Pixuna virus. In a
25 specific embodiment, the replicon elements are derived from VEEV.

As used herein, a sequence or replicon element is "derived from" a particular alphavirus (e.g. VEEV) if the sequence or replicon element corresponds to the wild-type sequence or replicon element of said alphavirus (e.g. VEEV), or has significant sequence identity thereto. In some embodiments, a sequence or replicon element is
30 "derived from" a particular alphavirus (e.g. VEEV) if the sequence has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or at least 100% sequence identity to the corresponding wild-type sequence or replicon element of said alphavirus (e.g. VEEV).

The term "heterologous" as used herein in reference to a nucleic acid sequence, refers
35 to a sequence that is from a different source than the nucleic acid sequence to which it is juxtaposed or operably linked. For example, a heterologous sequence

incorporated in a replicon RNA as described herein, refers to a nucleic acid sequence encoding a protein that is from a different source to the virus (e.g. alphavirus) from which the replicon is derived. A promoter operably linked to a coding sequence to which it is not operably linked in its natural state, is referred to as a "heterologous promoter".

The replicon RNA hereby provided is an alphavirus replicon RNA. An "alphavirus replicon" or "alphavirus replicon RNA" is a replicon derived from an alphaviral genome. In some embodiments, the alphaviral replicon is devoid of at least a portion of the sequence encoding the alphaviral structural proteins. In some embodiments, the replicon further comprises a heterologous nucleic acid, e.g., a gene of interest (GOI), also referred to as heterologous sequence which may be derived from a wide variety of sequences derived from any desired source. For example, in an alphavirus replicon RNA, the alphavirus autologous sequence encoding its structural proteins is replaced by the one or more GOI or heterologous sequences. Each GOI may be placed under the control of a dedicated (subgenomic) promoter or other control element as known to the person of skill in the art.

In some embodiments, the replicon RNA contains the following elements in said order (5'>3'): a 5' Cap, a 5'UTR sequence, sequences which encode biologically active non-structural proteins nsP1, nsP2, nsP3 and nsP4 (catalysing the RNA amplification), one or more heterologous sequences each of which may be operably linked to a subgenomic promoter or IRES, a 3'UTR sequence, and a poly(A) tail.

In some embodiments, the alphavirus replicon RNA contains the following elements in said order (5'>3'): a 5' Cap, a 5'UTR sequence, sequences which encode biologically active alphavirus non-structural proteins nsP1, nsP2, nsP3 and nsP4 (catalysing the RNA amplification), one or more heterologous sequences each of which may be operably linked to a subgenomic promoter or IRES, a 3'UTR sequence, and a poly(A) tail. In said embodiments, the nsP3 is an alphaviral nsP3 as described herein.

The present disclosure provides a modified form of the alphaviral non-structural protein 3 (nsP3), i.e. an alphaviral nsP3 comprising at least one mutation. As used herein, the term "modified" with respect to the alphaviral nsP3 described herein refers to the introduction of at least one single amino acid mutation, such as single amino acid substitution.

The VEEV nsP3 in its native form as referred to herein comprises the amino acid sequence of SEQ ID NO 1 (i.e. wild-type VEEV nsP3). It will be appreciated that wild-type VEEV nsP3 may have the longer amino acid sequence of SEQ ID NO 17 as a

result of stop codon read through. It will also be understood that nsP3 can be formed by proteolytic cleavage of the entire nsP1234 protein or by cleavage of nsP123, depending on the stop codon read through at the end of nsP3. The first amino acid of SEQ ID NO 1 (or SEQ ID NO 17) corresponds to position 1330 of the amino acid sequence of the full wild-type VEEV nsP1234 polyprotein. Such stopcodon may also (intentionally) be removed. Accordingly, in some embodiments, the nsP3 comprises a further mutation wherein the stop codon at position equivalent to position 551 of seq id 18 is mutated to an arginine thereby removing such stopcodon resulting in readthrough by default.

10 The alphaviral nsP3 disclosed herein is an alphaviral nsP3 which is modified at least by a mutation at a position equivalent to amino acid residue 167 of the wild-type VEEV nsP3 sequence of SEQ ID NO 1. Amino acid residue 167 of SEQ ID NO 1 corresponds to position 1496 of the amino acid sequence of the full wild-type VEEV nsP1234 polyprotein.

15 An "amino acid position equivalent to amino acid position 167 of the wild-type VEEV nsP3 sequence of SEQ ID NO 1" can be determined by any method of amino acid sequence alignment known in the art, such as BLAST or FastA protein alignment.

A "mutation" as used herein refers to a single amino acid substitution in a protein, or a single nucleotide substitution mutation in RNA.

20 In some embodiments, the alphavirus from which the nsP3 sequence is derived from an alphavirus selected from VEEV (such as TC-83 VEEV or Trinidad donkey virus), Everglades virus, Tonate virus, Mucambo virus Cabassou virus, Mosso das pedras virus, Rio Negro virus, and Pixuna virus and variants thereof. The wild-type nsP3 sequences of these alphaviruses are homologous to, and have high sequence similarity to, the wild-type VEEV nsP3 sequence.

25 It follows that, the alphaviral nsP3 disclosed herein may be an alphaviral nsP3 selected from VEEV (such as TC-83 VEEV or Trinidad donkey virus), Everglades virus, Tonate virus, Mucambo virus Cabassou virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus and variants thereof, modified at least by a mutation at a position equivalent to amino acid residue 167 of the wild-type VEEV nsP3 sequence of SEQ ID NO 1.

30 It will be appreciated that the alphaviral nsP3 disclosed herein may be derived from any alphavirus wherein the wild-type alphaviral nsP3 sequence is homologous to the wild-type VEEV nsP3 sequence. In some embodiments, the alphavirus from which the nsP3 is derived may have a wild-type nsP3 sequence that has at least 80%, at least

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85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% sequence identity to the wild-type VEEV nsP3 sequence of SEQ ID NO 1. In some embodiments, the alphavirus from which the nsP3 is derived may have a wild-type nsP3 sequence that has at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% sequence similarity to the wild-type VEEV nsP3 sequence of SEQ ID NO 1, optionally wherein the similarity-scoring matrix is a BLOSUM45 matrix.

In some embodiments, the alphaviral nsP3 is a VEEV nsP3 comprising a mutation at amino acid position 167.

For the purpose of the present disclosure, the alphaviral nsP3 mutation at position equivalent to position 167 of the wild-type VEEV nsP3, is a single amino acid substitution. In some embodiments, the amino acid as occurring in the wild-type sequence of the alphaviral nsP3 at the position equivalent to position 167 of the wild-type VEEV nsP3 is replaced by an amino acid other than isoleucine or leucine, or, other than isoleucine, leucine and valine.

In some embodiments, the alphaviral nsP3 comprises a methionine (Met) residue at amino acid position equivalent to amino acid position 167 of wild-type VEEV nsP3. In embodiments wherein the alphaviral nsP3 is a VEEV nsP3, the mutation at amino acid position 167 may be I167M. In some embodiments, the VEEV nsP3 has an amino acid sequence of SEQ ID NO:2. In some embodiments, the VEEV nsP3 has an amino acid sequence of SEQ ID NO:18 (as a result of stop codon read through). The first amino acid of SEQ ID NO:2 or SEQ ID NO:18 corresponds to position 1330 of the amino acid sequence of the full VEEV nsP1234 polyprotein.

In some embodiments, the alphaviral nsP3 has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO:2, providing the amino acid at position 167 is Met. In some embodiments, the alphaviral nsP3 has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO:18, providing the amino acid at position 167 is Met.

In a further aspect, a replicon RNA is provided comprising a sequence encoding alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, wherein said alphaviral nsP3 is an alphaviral nsP3 described herein. Any replicon RNA construct encoding alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4 may benefit from the inclusion of a sequence encoding the nsP3 described herein. Accordingly, replicon RNAs are provided encoding an alphaviral nsP3 as described herein. For example,

recombinant alphaviral replicon RNAs wherein the sequence encoding the naturally occurring nsP3 is replaced by a sequence encoding the nsP3 as described herein.

The sequence encoding alphaviral nsP3 described herein may be incorporated in replicons derived from other viruses (e.g. alphaviruses other than VEEV) to obtain an effect on the replicon functionality similar to that shown herein for VEEV derived replicons. For example, a sequence encoding an alphaviral nsP3 described herein may be incorporated in replicons derived from Sindbis virus.

The alphaviral nsP3 as provided herein confers particular characteristics to the overall replicon (e.g. alphavirus replicon) as illustrated in the present Examples, in particular reduced RNA transcription (resulting in reduced innate immune response and/or reactogenicity), whilst inducing broad adaptive immune response against an encoded GOI (including both humoral and cellular mediated immune (CMI) response (including CD8+ T-cell induction)). A reduced innate immune response may be demonstrated, for example, by the production of fewer pro-inflammatory transcripts related to the IFN-alpha pathway. Reduced reactogenicity may be demonstrated, for example, by measurement of serum cytokine levels (e.g. IFN- γ , CXCL-1 (KC), TNF- α , CCL2 (MCP-1), CXCL10 (IP10), IFN- α and IL-6). Vaccines that elicit a strong CMI response are particularly sought after for pathogens that can persist or hide inside host cells, such as certain viruses and certain bacteria (e.g., Chlamydia trachomatis and Mycobacterium tuberculosis). A balanced CMI and humoral (antibody-mediated) response is expected to provide robust and durable immunity.

The non-structural proteins

As defined herein, a replicon RNA is an RNA molecule that, contrary to conventional mRNA, contains all of the genetic information required for directing its own amplification or self-replication within a permissive cell.

The replicon RNA may be an alphavirus replicon RNA. Typically, for alphaviral replicon RNA, the non-structural proteins 1–4 (nsP 1–4) form the RNA-dependent RNA polymerase (RDRP), which is responsible for replication of the saRNA thereby producing copies of the saRNA. Multiple copies of the subgenomic RNA are hence produced from each saRNA originally delivered. This leads to translation of many more copies of the heterologous coding sequence when compared to a non-self amplifying RNA.

The replicon RNA may comprise sequences encoding each of the alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, optionally independently derived from

one or more alphaviral genomes, wherein the nsP3 is an alphaviral nsP3 described herein.

The replicon RNA may comprise a sequence encoding the alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, wherein said sequence is modified at least by a
5 modification at the nucleotide position equivalent to position 4488 of the sequence encoding wild-type VEEV nsPs 1-4 of SEQ ID NO:3. As used herein, "nsPs 1-4" may refer to the nsP1234 polypeptide.

An "nucleotide position equivalent to amino acid position 4488 of the sequence encoding wild-type VEEV nsPs 1-4 of SEQ ID NO:3" can be determined by any method
10 of nucleotide sequence alignment known in the art, such as BLAST or FastA nucleotide alignment.

The alphavirus from which the sequence encoding the non-structural proteins (nsP1, nsP2, nsP3 and nsP4) is derived may be selected from VEEV (such as TC-83 VEEV or Trinidad donkey virus), Everglades virus, Tonate virus, Mucambo virus Cabassou
15 virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus and variants thereof.

In some embodiments, the sequence encoding the non-structural proteins (nsP1, nsP2, nsP3 and nsP4) is derived from VEEV. The wild-type sequence encoding VEEV nsPs1-4 is provided in SEQ ID NO:3.

As defined elsewhere herein, a sequence may be "derived from" a particular
20 alphavirus (e.g. VEEV) if the sequence corresponds to the wild-type sequence of said alphavirus (e.g. VEEV), or has significant sequence identity thereto, for example has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity thereto.

In some embodiments, the sequence encoding the alphaviral nsPs1-4 has a
25 nucleotide sequence of SEQ ID NO:4 (i.e. the sequence encoding VEEV nsPs1-4 modified by an A4488G mutation). In some embodiments, the sequence encoding the alphaviral nsPs1-4 has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO:4, providing the sequence encodes a nsP3 comprising a Met residue at the
30 position equivalent to residue 167 of SEQ ID NO 1.

Alternatively, the sequences encoding each of the non-structural proteins (nsP1, nsP2, nsP3 and nsP4) may be independently derived from one or more alphaviruses selected from VEEV (such as TC-83 VEEV or Trinidad donkey virus), Everglades virus, Tonate virus, Mucambo virus Cabassou virus, Mosso das pedras virus, Rio Negro
35 virus, Pixuna virus, and variants thereof.

In some embodiments, the non-structural proteins 1, 2 and/or 4 correspond to their native (wild type) counterparts. In some embodiments, the non-structural proteins nsP1, nsP2 and nsP4 are wild type.

5 In alternative embodiments, sequences encoding modified forms of the non-structural proteins, for example modified forms of nsP1, nsP2 and/or nsP4, are used. Further modifications of the native nsPs (other than the nsP3 mutation provided herein) have been described in the art that may also be applied to the nsP3 or replicon as described herein (see for example: Li, Y. et al. In vitro evolution of enhanced RNA replicons for immunotherapy. Sci Rep 9, 6932 (2019); LaPointe A.T. et al. Increasing
10 the capping efficiency of the Sindbis Virus nsP1 Protein negatively affects viral infection. mBio 9(6):e02342-18 ((2018)), e.g. to increase transcription or translation efficiency, or to create a favourable immune response (innate and/or adaptive).

In some embodiments, the replicon RNA comprises sequences encoding the non-structural proteins nsP1, nsP2 and/or nsP4 derived from VEEV. The wild-type
15 sequence encoding VEEV nsP1 is provided in SEQ ID NO:5. The wild-type sequence encoding VEEV nsP2 is provided in SEQ ID NO:6. The wild-type sequence encoding VEEV nsP4 is provided in SEQ ID NO:9.

The wild-type RNA sequence encoding VEEV nsP3 is provided in SEQ ID NO:7, and the RNA sequence coding for VEEV nsP3 modified by a I167M mutation is provided
20 in SEQ ID NO:8. The RNA sequence of SEQ ID NO: 7 and SEQ ID NO: 8 includes the stop codon "UGA" near the 3' end, as well as the subsequent read through. Therefore, SEQ ID NO: 7 may encode SEQ ID NO: 1 or SEQ ID NO: 17 depending on stop codon read through. Similarly, SEQ ID NO:8 may encode SEQ ID NO: 2 or SEQ ID NO 18, depending on stop codon read through. It will be understood that RNA sequences
25 lacking the stop codon read through, but still encoding the wild-type VEEV nsP3 of SEQ ID NO:1 or the I167M mutated VEEV nsP3 of SEQ ID NO:2, may be incorporated into the replicon RNA described herein.

In some embodiments, the RNA sequence encoding the alphaviral nsP3 has at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least
30 98%, or at least 99% sequence identity to SEQ ID NO:8, providing the sequence encodes a nsP3 comprising a Met residue at the position equivalent to residue 167 of SEQ ID NO 1.

It follows that, the sequence encoding nsPs1-4 may comprise SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:8 and SEQ ID NO:9 (i.e. encoding wild-type VEEV nsP1, 2 and 4,
35 and nsP3 modified by a I167M mutation).

In some embodiments, the non-structural proteins nsP1, nsP2 and nsP4 are derived from VEEV and comprise a further mutation in nsP2, such as Q739L (see Petrakova O et al. Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in Mammalian cells. J Virol. 2005 Jun;79(12):7597-608). Any mutation in the non-structural proteins known to the skilled person to confer demonstrated characteristics to the replicon RNA (such as further adaptive mutations) may be considered for combination of the nsP3 mutation at position 167 described herein.

For replicon RNAs provided herein, the replicon elements of a replicon RNA from any virus (e.g. alphavirus) may be used as the basis from which to derive the replicon sequences, such as the alphaviruses described above from which the sequences encoding the nsPs may be derived.

Suitable wild-type alphavirus sequences are well-known and are available from sequence depositories, such as the American Type Culture Collection, Rockville, Md. Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC 30 VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo 5 virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Trinita (ATCC VR-469), Una (ATCC VR-374), Venezuelan equine encephalomyelitis (ATCC VR-69, ATCC VR-923, ATCC VR-1250 ATCC VR10 1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375). In a particular embodiment, the alphavirus is a Venezuelan Equine Encephalitis Virus (VEEV). In a more particular embodiment, the alphavirus is a live attenuated Venezuelan Equine Encephalitis Virus (VEEV), such as strain TC-83.

5' Cap

The replicon RNA hereby provided may have at least one 5' terminal cap. As used herein, a "5'-cap" is an entity, typically a modified nucleotide entity, which generally "caps" the 5'-end of a mature mRNA. A 5'-cap may typically be formed by a modified nucleotide, particularly by a derivative of a guanine nucleotide. Preferably, the 5'-cap is linked to the 5'-terminus via a 5'-5'-triphosphate linkage. A 5'-cap may be

methylated, e.g. m7GpppN, wherein N is the terminal 5' nucleotide of the nucleic acid carrying the 5'-cap, typically the 5'-end of an RNA. Further examples of 5'cap structures include glyceryl, inverted deoxy abasic residue (moiety), 4', 5' methylene nucleotide, 1-(beta-D-erythrofuransyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide, 1,5-anhydrohexitol nucleotide, L- nucleotides, alpha-nucleotide, modified base nucleotide, threo-pentofuransyl nucleotide, acyclic 3',4'-seco nucleotide, acyclic 3,4-dihydroxybutyl nucleotide, acyclic 3,5 dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety, 3'-3'-inverted abasic moiety, 3'-2'-inverted nucleotide moiety, 3'-2'-inverted abasic moiety, 1,4-butanediol phosphate, 3'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 3'phosphorothioate, phosphorodithioate, or bridging or non-bridging methylphosphonate moiety. Further modified 5'-cap structures which may be used in the context of the replicon RNA hereby provided are cap1 (methylation of the ribose of the adjacent nucleotide of m7GpppN), cap2 (methylation of the ribose of adjacent and the 2nd nucleotide downstream of the m7GpppN), cap3 (methylation of the ribose of the adjacent, 2nd and 3rd nucleotide downstream of the m7GpppN), cap4 (methylation of the ribose of the adjacent, 2nd, 3rd and 4th nucleotide downstream of the m7GpppN), ARCA (anti-reverse CAP analogue), modified ARCA (e.g. phosphothioate modified ARCA), inosine, N1-methyl-guanosine, 2'-fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine. A specifically preferred 5'-cap is the CleanCap structure, offered by TriLink BioTechnologies.

The 5' untranslated region (5'UTR)

Typically, the replicon RNA hereby provided comprises a 5'UTR. The 5'UTR is a sequence of nucleotides at the 5' end of a mRNA molecule located upstream of the initiation codon and thus not translated into protein. The 5'UTR may play several roles in gene expression and/or post-transcriptional regulation including regulation of translation initiation and modulation of protein expression.

In some embodiments, the 5'-UTR used is heterologous to the heterologous coding sequence, such as derived or retained from an alphaviral genome from which the replicon is derived. Even if 5'-UTR's derived from naturally occurring genomes are preferred, also synthetically engineered 5-UTR's may be used in the context of the present disclosure.

The subgenomic promoter/IRES

The replicon RNA hereby provided may comprise one or more heterologous sequences, each of which being operably linked to one or more promoter and/or IRES

elements, optionally wherein the promoter is a subgenomic promoter. Each heterologous sequence may encode one or more heterologous gene products.

In some embodiments, the replicon sequences otherwise encoding the viral structural proteins (e.g. alphaviral structural proteins) are replaced by the one or more
5 heterologous sequences, thereby placing the heterologous sequence(s) under the control of the native viral promoter (e.g. alphaviral promoter). Alternatively, one or more of the heterologous sequences may be placed under the control of a heterologous regulatory element such as a heterologous subgenomic promoter.

In some embodiments, the one or more heterologous sequences are operably linked
10 to one or more promoters (e.g. a subgenomic promoter). Two or more heterologous sequences may each be operably linked to different promoters (e.g. subgenomic promoters). Alternatively, two or more heterologous sequences may be operably linked to the same promoter (e.g. subgenomic promoter).

As used herein, the term "subgenomic promoter" refers to those sequences that
15 constitute a functional element required for production of subgenomic RNA species. A subgenomic promoter can drive the expression of genes using RNA as template; the subgenomic promoter is recognized by an RNA-dependent RNA polymerase, which may be a viral RNA replicase. The promoter itself may be a composite of segments derived from more than one source, naturally occurring, autologous or
20 heterologous to the source of the replicon, or, synthetic. It should be noted that a subgenomic promoter is located in relation to a subgenomic RNA species or a particular gene whose transcription it initiates, and it is functionally recognized by an RNA-dependent RNA polymerase (or viral RNA replicase) when it is contained within an RNA molecule of the proper (-) polarity. The (-) sense RNA molecule containing
25 the functional copy of the subgenomic promoter, which comprises the functional units of core promoter and activating domain, can be synthesized by RNA-dependent RNA polymerase using a (+) sense RNA molecule as template, or it may have been synthesized by (cellular) RNA polymerase II as a transcript initiated by a polII promoter.

30 In some embodiments, the one or more heterologous sequences are operably linked to one or more IRES elements. Two or more heterologous sequences may each be operably linked to a different IRES. Alternatively, two or more heterologous sequences may be operably linked to the same IRES.

Thus, an internal ribosome entry site (IRES) sequence may be incorporated into the
35 replicon RNA to control the expression of a heterologous coding sequence. An IRES is an RNA element that in its native form allows for translation initiation (ribosome

assembly) in a cap-independent manner. IRES elements have been used to modulate expression of a gene of interest administered through mRNA.

In some embodiments, one or more heterologous sequences are operably linked to one or more promoters (e.g. a subgenomic promoter) and one or more heterologous
5 sequences are operably linked to one or more IRES elements.

Heterologous sequence (also referred to as gene of interest (GOI)) and methods of use

Typically, the saRNA or replicon RNA described herein is considered useful as a means for tailored expression of a product encoded by a heterologous sequence placed under
10 the control of a subgenomic promoter, IRES or equivalent. Accordingly, certain embodiments relate to the use of the replicon RNAs to express the encoded product in a cell.

Each heterologous sequence of a replicon RNA provided herein may encode one or more heterologous gene products. The heterologous gene product may, for example,
15 be a heterologous protein, oligonucleotide or polynucleotide, optionally wherein the oligonucleotide or polynucleotide is RNA (e.g. siRNA, gRNA). In this context, the gene product is heterologous to the virus from which the replicon RNA is derived.

In some embodiments, one or more of the heterologous sequences encodes a heterologous protein, for example a heterologous protein that is derived from a
20 pathogen (e.g. bacterial, viral, fungal, protozoan or multi-cellular parasitic pathogen), allergen or tumor.

In some embodiments, the heterologous protein is an antigenic protein. For example, in some embodiments, the replicon RNA serves as a vector to deliver pathogen derived antigens. Any protein derived antigen known in the art can be considered for
25 delivery by the replicon RNA, the antigen being encoded by the/a heterologous sequence.

In some embodiments, the replicon RNA serves as a vector to deliver tumor derived antigens. Any tumor-associated antigen (TAA) or tumor specific antigen (TSA) made available in the art can be considered for the heterologous sequence of the replicon
30 RNAs provided herein.

The replicons as described herein are particularly useful for the delivery of an antigen (particularly a pathogen derived antigen) for prophylactic or therapeutic vaccination purpose.

Alternatively, the replicons are considered useful for substitution therapy in light of
35 the sustained expression profile as determined in the *in vivo* experiments reported

herein. Accordingly, in some embodiments, the heterologous protein is a therapeutic protein. Such therapeutic protein may be a protein otherwise endogenic to the individual but which the individual fails to produce at sufficient levels or for which the endogenous protein is expressed in a non-functional form. The replicon RNAs of the invention can be used to deliver the (correct) protein in protein replacement therapy.

3' untranslated region (3'UTR)

The replicon RNA disclosed may comprise a 3' UTR.

A 3'-UTR is typically the part of an mRNA, which is located between the protein coding region (i.e. the open reading frame) and the 3'-terminus of the mRNA. A 3'-UTR of an mRNA is not translated into an amino acid sequence. The 3'-UTR sequence is generally encoded by the gene, which is transcribed into the respective mRNA during the gene expression process. In the context of the present disclosure, a 3'-UTR corresponds to the sequence of a replicon, which is located 3' to the stop codon of the heterologous coding sequence, preferably immediately 3' to the stop codon of the heterologous coding sequence, and which extends to the 5'-side of the 3'-terminus of the replicon or of the poly(A) tail, preferably to the nucleotide immediately 5' to the poly(A) tail. The term "corresponds to" means that the 3'-UTR sequence may be an RNA sequence, such as in the replicon sequence used for defining the 3'-UTR sequence, or a DNA sequence, which corresponds to such RNA sequence. Preferably, the 3'-UTR used according to the present disclosure is heterologous to the heterologous coding sequence, such as derived or retained from the alphaviral genome from which the replicon is derived. Even if 3'-UTR's derived from naturally occurring genomes are preferred, also synthetically engineered UTR's may be used in the context of the present disclosure.

Poly(A) tail

The term "3'-poly(A) tail" or "poly(A) tail" typically refers to a stretch of adenine nucleotides at to the 3'-end of an mRNA. In the context of the present disclosure, the term refers to such poly(A) tail at the 3' end of the 3'UTR of the replicon. It can, in some instances, be as short as 20 nucleotides or comprise up to about 500 adenine nucleotides. In some cases, the length of the 3'-poly(A) tail may be an essential element with respect to the stability of the RNA strand. Said length can be of up to about 400 adenine nucleotides, e.g. from about 20 to about 400, preferably from about 30 to about 400, more preferably from about 30 to about 300, even more preferably from about 30 to about 250. In embodiments, the poly(A) tail has about 30, about 40, about 50 about 60, about 70, about 80, about 100 or about 120 nucleotides. The poly(A) tail may be a variant such as a segmented poly(A) tail,

characterized in that it consists of at least two A-containing elements each defined as a nucleotide sequence consisting of 20–60 adenosines separated by a spacer element of differing length (see for example Trepotec Z, Geiger J, Plank C, Aneja MK, Rudolph C. Segmented poly(A) tails significantly reduce recombination of plasmid DNA without affecting mRNA translation efficiency or half-life. RNA. 2019 Apr;25(4):507-518).

In some embodiments, the replicon RNA as disclosed herein is further modified in ways that function independently to the modification of the alphavirus nsP3 as described herein, nevertheless potentially further improving the overall replicon functionality. A number of such modifications are known to the skilled person and thereby contemplated for the replicon RNA disclosed herein.

Accordingly, in some embodiments, codon optimisation is applied to the nucleotide sequence of the replicon RNA. Depending on the codon optimisation algorithm used, characteristic of the replicon RNA such as overall stability, further reduction of innate sensing etc. may be achieved.

In some embodiments, codon optimisation of the replicon RNA is according to the algorithm described by He Zhang et al. (Nature (2023) 621, 396-403). Alternative codon optimisation tools are equally available and known to the skilled person.

Also the use of modified nucleosides in the replicon RNA described herein is contemplated. In some embodiments, one or more modified nucleosides are used in the manufacture or design of the replicon RNA selected from the group comprising pseudouridine, N1-methyl-pseudouridine, 5-methyl-uridine, pseudocytidine, N1-methyl-pseudocytidine and 5-methyl-cytidine. In one embodiment, the modified nucleoside is independently selected from N1-methyl-pseudouridine or 5-methyl-cytidine. In one embodiment, substantially 50%, 60%, 70%, 80%, 90% or 100% of cytidine and/or uridine included in the replicon RNA are modified cytidine (e.g. 5-methyl-cytidine) and/or modified uridine (e.g. N1-methyl-pseudouridine) respectively. In one embodiment, 100% of cytidine is modified cytidine.

Methods of making

The replicon RNAs described herein may be obtained by any means known in the art for the manufacture of RNA molecules. Typically, synthetic RNA molecules are manufactured by in vitro transcription (IVT). IVT requires a linear DNA template containing a dedicated promoter to initiate the transcription of the target RNA sequence.

Thus, the disclosure provides a DNA template encoding a replicon RNA as described herein, in particular a replicon RNA as defined by the claims. The DNA template is typically obtained by linearisation of a plasmid comprising the promoter and target RNA encoding sequence. Alternatively, the template may be obtained using other techniques known in the art to provide synthetic DNA templates suitable for use in IVT, techniques such as but not limited to PCR, rolling circle amplification (RCA) or synthetic DNA fragment synthesis. In some embodiments, the DNA template is provided for use in a method of making the modified replicon RNA described herein, such as IVT. Common promoters for RNA transcription from the DNA template include the T7, SP6, or T3 promoters. The IVT reaction mixture further comprises ribonucleotide triphosphates, the appropriate phage RNA polymerase for the promoter present in the DNA template (e.g. T7, SP6 or T3 RNA polymerases, including their modified variants described in the art), and a buffer system tailored to the RNA polymerase used. An RNase inhibitor may further be included in the reaction mixture. It is within the artisan's skill to determine the exact conditions to be used in the IVT reaction (inter alia depending on the amount of RNA needed for a specific application as well as length and nature of the target RNA molecule). Various RNA synthesis kits are available, some of which optimized for long RNA transcripts. Examples include HiScribe T7 High Yield RNA Synthesis Kit (New England BioLabs).

Typically, the RNA molecule is protected by a 5'-cap. 5'-capping of RNA may be completed concomitantly during the IVT reaction using the following chemical RNA cap analogs to generate the 5'-guanosine cap structure according to manufacturer protocols: 3'-O-Me-m7G(5')ppp(5') G [the ARCA cap]; G(5')ppp(5')A; G(5')ppp(5')G; m7G(5')ppp(5')A; m7G(5')ppp(5')G (New England BioLabs). 5'-capping of modified RNA may also be completed post-transcriptionally using a Vaccinia Virus Capping Enzyme to generate the "Cap 0" structure: m7G(5')ppp(5')G (New England BioLabs). Cap 1 structure may be generated using both Vaccinia Virus Capping Enzyme and a 2'-O methyl-transferase to generate: m7G(5')ppp(5')G-2'-O-methyl. Cap 2 structure may be generated from the Cap 1 structure followed by the 2'-O-methylation of the 5'-antepenultimate nucleotide using a 2'-O methyl-transferase. Cap 3 structure may be generated from the Cap 2 structure followed by the 2'-O-methylation of the 5'-preantepenultimate nucleotide using a 2'-O methyl-transferase. Enzymes may be derived from a recombinant source. Alternatively, 5'-capping of polynucleotides may be achieved by co-transcriptional capping using m2 7,3'-oGpppG or m2 7,2'-oGpppG ARCA (CellScript Inc), CleanCap technology such as CleanCap AU (TriLink Biotechnologies LLC), 5'- phosphorothiolate cap analogs (Univ. Warszawski), or by use of alternative cap analogues.

Methods Of Expression, Nanoparticles and Cells

The disclosure provides a method of expressing one or more heterologous gene products in a cell, comprising: introducing a replicon RNA as described herein into a cell; and expressing the one or more heterologous gene products encoded by said replicon in the cell. In some embodiments, one or more of the heterologous gene products is a heterologous protein. In some embodiments, one or more of the heterologous gene products is a heterologous RNA (e.g. an siRNA or gRNA). In some embodiments, the heterologous gene product is a heterologous protein that is an antigenic protein, in particular an antigenic protein which when expressed by the replicon RNA induces a prophylactic and/or therapeutic immune response at least in part due to a broad humoral and cell-mediated (including CD8⁺ and/or CD4⁺ T-cell response) similar to the one reported for the constructs reported herein.

The replicon RNAs described herein are amenable for delivery to cells in an in vitro cell culture as well as in vivo to a subject in need thereof by any means known in the art for the delivery of RNA. Viral particle like packaging has been described. Nonviral formulations including cationic lipids, LNPs, polymers, and protamine sulphate, as well as physical methods such as electroporation may be used to deliver RNA therapeutics.

Methods known in the art of mRNA for cellular transfection and/or (therapeutic) administration of mRNA to an individual are equally considered for the delivery of the modified replicon RNA described herein, such as the methods referred to by Xiang Li et al. (Theranostics 2024, Vol. 14, Issue 2, pages 738-760) or Paunovska K, et al. Drug delivery systems for RNA therapeutics. Nat Rev Genet. 2022 May;23(5):265-280. These typically include cationic moieties that allow both association of the lipids with the negatively charged RNA, thereby protecting the RNA for RNase activity, as well as efficient uptake in the cell including endosomal escape. At the same time, the inclusion of the nsP1-4 replicon sequence in saRNAs makes them much longer than their conventional mRNA counterparts which is important for formulation. Over the past years several new or dedicated approaches aimed at improving the delivery of saRNAs have been described amongst others by Bloom K. et al. (Self-amplifying RNA vaccines for infectious diseases. (2021) *Gene Ther* **28**, 117–129) and Liu Y et al. (Liu, Y.; Li, Y.; Hu, Q. Advances in saRNA Vaccine Research against Emerging/Re-Emerging Viruses. *Vaccines* 2023, *11*, 1142) .

The disclosure provides a nanoparticle comprising a replicon RNA as described herein. In some embodiments, the nanoparticle is a lipid nanoparticle (LNP). LNP

formulations known to the skilled person, such as formulations used for mRNA comprising vaccines, typically comprise an ionisable cationic lipid, a helper or structural lipid such as DSPC or a phosphatidyl choline based analogue, a polyethylene glycol conjugated lipid and cholesterol. In some embodiments, the replicon RNA is formulated using lipid nanoparticle technology using a ionisable cationic lipid such as described in WO2023078946, WO2023078950 or WO2023078954 (or, US18/705454, US18/705479, or US18/705396 respectively, which are incorporated herein by reference). In some embodiments, the nanoparticle is a virus like particle (VLP). In some embodiments, the nanoparticle is a polymer based particle (such as polymer-based vehicles referred to in Table 2 of Xiang Li et al. Theranostics 2024, Vol. 14, Issue 2, or similar thereto).

In some embodiments of the method, the replicon RNA is introduced into the cell by direct transfection.

The disclosure also provides a cell comprising a replicon RNA as described herein. The cell may be a mammalian cell, such as human cell. The cell may lack native expression of the one or more heterologous gene products (e.g. a protein) encoded by the replicon RNA. The cell may have defective expression of the one or more heterologous gene products (e.g. a protein) encoded by the replicon RNA.

Pharmaceutical Compositions

The disclosure provides a pharmaceutical composition comprising a replicon RNA as described herein, a nanoparticle comprising a replicon RNA as described herein, or a cell comprising a replicon RNA as described herein.

In embodiments where the pharmaceutical composition comprises a cell comprising a replicon RNA, one or more heterologous gene products encoded by the replicon RNA are typically expressed in said cell. For instance, the transcription or translation product(s) encoded by the heterologous sequence of the replicon RNA may be expressed in said cell. In embodiments where the heterologous gene product is a protein, the cell may present the protein on the cell surface. In some embodiments, a pharmaceutical composition comprising one or more modified replicon RNAs provides *in situ* release of the translation product of the one or more heterologous sequences. In some embodiments, the heterologous sequence encoded by the replicon RNA is a chimeric antigen receptor (CAR) and the cell an immune cell, such as a human immune cell or immune cell isolated from a patient. In said embodiment, the pharmaceutical composition is for use in CAR-based therapies, such as CAR-T cell therapy or CAR-NK cell therapy. Typically, CAR-based therapies are designed to treat

cancer. Alternatively, CAR-based therapies for which the replicon RNA described herein is provided is also for use in the treatment of certain autoimmune diseases.

In some embodiments, replicon RNA is provided that encodes the CAR constructs as described in US20240115606 (herein incorporated by reference), as well as immune
5 cells, such as NK-cells, comprising such replicon RNA, and a pharmaceutical composition comprising such immune cells and/or replicon RNA.

Suitably formulated in a pharmaceutical composition, the replicon RNAs described herein can be used in methods of treatment of a disease in a subject, either as a prophylactic or therapeutic. The subject is preferably mammalian, more preferably a
10 human.

Alternatively, a replicon RNA as described herein, a nanoparticle as described herein, a cell as described herein, or a pharmaceutical composition as described herein may be for use in a method of inducing an immune response in an individual, or for use in vaccination. As explained elsewhere herein, the alphaviral nsP3 described herein
15 produces a broad adaptive immune response (including robust humoral and cell mediated responses (including CD8+ T cell responses)) which is particularly favourable for the prophylactic treatment of a disease in the subject.

Alternatively, a replicon RNA as described herein, a nanoparticle as described herein, a cell as described herein, or a pharmaceutical composition as described may be for
20 use in therapy in treating a gene expression disorder in a patient.

Most common routes for administration of such pharmaceutical composition are by injection, such as intradermal (ID), subcutaneous (SC), intramuscular (IM), intranodal (IN), and intravenous (IV). In a particular embodiment, the pharmaceutical composition comprising the suitably formulated replicon RNA is
25 administered IM. Other routes, including mucosal administration routes such as intranasal injection, intratracheal instillation, and intravaginal injection, and, intratumoral injection described for mRNA delivery (such as reviewed by Zeng C, et al. Curr Top Microbiol Immunol. 2022;440:71-110) are equally considered for the replicon RNAs disclosed herein.

30 The replicon RNA pharmaceutical compositions described herein may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would
35 be administered to a subject or a convenient fraction of such a dosage such as, for

example, one-half or one-third of such a dosage. The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the disclosure will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) of one or more of the replicon RNAs described herein. In addition to the replicon RNA component(s), a pharmaceutical composition of the disclosure may further comprise one or more additional pharmaceutically active agents. It is further contemplated that controlled- or sustained-release replicon RNA formulations may be made using known dosage forms and techniques.

Exemplary dosage forms suitable for parenteral administration (e.g. subcutaneous, intradermal, subdermal, intramuscular, intravenous, intraperitoneal, intra-articular, and infusion) include, for example, solutions, suspensions, dispersions, emulsions, and the like. They may also be manufactured in the form of sterile solid compositions (e.g. lyophilized composition), which can be dissolved or suspended in sterile injectable medium immediately before use. They may contain, for example, suspending or dispersing agents known in the art.

Methods of treatment

The disclosure also includes methods of treating a disease or condition or for inducing an immune response in a subject in need thereof, comprising administering a pharmaceutical composition comprising any of the replicon RNAs described herein, thereby causing expression of the heterologous gene(s) of interest encoded by the replicon RNA in one or more cells, tissues, or organs of the human subject. In some aspects, the replicon RNA is administered in a therapeutically effective amount. In some embodiments, the therapeutically effective amount comprises or is an amount that causes expression of an amount of the heterologous protein(s) encoded by the replicon sufficient to prevent, reduce (severity of) or eliminate one or more symptoms of the disease or condition. In further embodiments, the methods are directed to treating a gene expression disorder in a human subject, optionally where the patient lacks the endogenous expression of the one or more heterologous gene product(s) (e.g. protein) encoded by the replicon RNA described herein, or where the endogenously expressed protein is dys- or malfunctional.

In some embodiments, the use of the replicon RNA in CAR-based therapies is provided. Accordingly, a method of treatment is provided comprising the step of administering a subject in need thereof of a therapeutically effective amount of, or,

number of immune cells comprising a replicon RNA encoding a chimeric antigen receptor. In further embodiments, such treatment is a cancer treatment, such as treatment of a blood tumor (such as ALL, DLBCL, and multiple myeloma), or, a solid tumor malignancy. In a further embodiment, the replicon RNA is for use in a method
5 of treatment as described in US 20240115606.

In other embodiments, the use of the replicon RNA in prophylactic treatment or vaccination is provided, thereby avoiding the occurrence, reducing the severity of, or, reducing the progress of a disease or symptoms of a disease. As used herein, the term "vaccination" refers to a method of treatment using a replicon RNA as described
10 herein to induce or enhance an immune response of the subject (receiving the treatment) against the heterologous protein(s) encoded by the replicon RNA. A therapeutically effective amount of the replicon RNA for vaccination therefore comprises or is an amount that causes expression of the heterologous protein(s) encoded by the replicon sufficient to induce an immune response sufficient to
15 prevent, reduce (severity of) or eliminate one or more symptoms of the disease or condition otherwise occurring.

In some embodiments, the disease or condition is caused by a pathogen and/or the condition is infection by a pathogen. Accordingly, in some embodiments, the replicon RNA comprises (a) heterologous gene(s) of interest that encode an antigen of a
20 pathogen. Antigens suitable for use in vaccination, i.e. known in the art to give rise to a protective immune response (e.g. neutralising antigen-specific antibodies and/or T-cells) are hereby contemplated, in particular for pathogens known to require a robust cellular immune response to prevent infection or reduce severity of the disease caused by infection.

25 The term "pathogen" refers to pathogenic biological material capable of causing disease in a subject, such as a mammal. Pathogens include microorganisms such as bacteria, unicellular eukaryotic organisms (protozoa), fungi as well as viruses.

The term "antigen", "antigenic peptide", "epitope", "immunogenic peptide" and the like are used interchangeably and refer to an antigenic determinant in a molecule
30 such as an antigen, i.e. to a part in or fragment of an immunologically active compound that is recognized by the immune system of the subject exposed to the antigen and causes the immune system of the subject to generate an antigen-specific immune response. In the context of the methods of vaccination described herein, the antigen is derived from the targeted pathogen, i.e pathogen against which the elicited
35 immune response is directed.

In some embodiments, the targeted pathogen is a virus, such as selected from viruses belonging to Coronaviruses, Orthomyxoviruses, Paramyxoviridae viruses, Pneumoviruses, Rubulaviruses, Paramyxoviruses, Metapneumoviruses and Morbilliviruses, Poxviridae, Orthopoxvirus such as Variola vera, Picornaviruses, 5 Enteroviruses, Rhinoviruses, Heparnaviruses, Cardioviruses, Aphthoviruses, Bunyavirus, Heparnaviruses, Filoviruses, Togaviruses, Flaviviruses, Pestiviruses, Hepadnaviruses, other hepatitis viruses, Rhabdoviruses, Caliciviridae, Retroviruses, Reoviruses, Parvoviruses, Herpesviruses, Papovaviruses and Adenoviruses. In further embodiments, the targeted virus from which the antigen is derived is known as a 10 respiratory virus such as influenza virus, respiratory syncytial virus, human metapneumovirus, rhinovirus, parainfluenza virus, coronavirus (e.g. SARS-CoV), adenovirus or bocavirus.

In some embodiments, the targeted pathogen is a bacteria chosen from *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, *Bordetella pertussis*, *Staphylococcus aureus*, *Clostridium tetani*, 15 *Corynebacterium diphtheriae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Helicobacter pylori*, *Escherichia coli*, *Bacillus anthracis*, *Yersinia pestis*, *Staphylococcus epidermis*, *Clostridium perfringens* or *Clostridium botulinum*, 20 *Legionella pneumophila*, *Coxiella burnetii*, *Brucella*, *Francisella*, *Neisseria gonorrhoeae*, *Treponema pallidum*, *Haemophilus ducreyi*, *Enterococcus faecalis* or *Enterococcus faecium*, *Staphylococcus saprophyticus*, *Yersinia enterocolitica*, *Mycobacterium tuberculosis*, *Rickettsia*, *Listeria monocytogenes*, *Vibrio cholerae*, *Salmonella typhi*, *Borrelia burgdorferi*, *Porphyromonas gingivalis*, *Klebsiella*.

25 In some embodiments, the targeted pathogen is a pathogen known as causing sexually transmitted infections (STIs), such as syphilis, Gonorrhoea, Chlamydia and Trichomoniasis, hepatitis B, herpes simplex virus (HSV), HIV or human papillomavirus (HPV). In some embodiments, the antigen is a Chlamydia antigen, for example as described in WO2014/146663 (hereby incorporated by reference for the 30 antigens described therein) or similar thereto.

In some embodiments, the targeted pathogen is a pathogenic parasite. In some embodiments, the pathogenic parasite is a parasitic protozoan and belongs to a genus selected from the group consisting of: *Plasmodium*, *Toxoplasma*, *Babesia*, *Eimeria*, *Theileria*, *Neospora*, *Sarcocystis*, *Leishmania*, and *Trypanosoma*. In some 35 embodiments, the parasite is a helminth, for example a nematode. In some embodiments, the parasitic helminth belongs to a genus selected from the group

consisting of: Ancylostoma, Necator, Brugia, Wuchereria, Loa, Mansonella, Trichinella, Trichuris, Ascaris, Anisakis, Dracunculus, Strongy 'hides, Haemonchus, Schistosoma and Fasciola. In some embodiments, the antigen is selected from a migration inhibitory factors (MIF) antigen as described in WO2015/144732 (hereby
5 incorporated by reference for the antigens described therein).

In some embodiments, the term "subject" relates to vertebrates, in particular mammals. In some embodiments, mammals are humans, non-human primates, domesticated animals such as dogs, cats, sheep, cattle, goats, horses etc., laboratory animals such as mice, rats, rabbits, guinea pigs etc., as well as animals in captivity
10 such as animals of zoos.

In some embodiments, the term "subject" also relates to non-mammalian vertebrates such as birds (for example domesticated birds such as chicken, ducks, geese or turkeys) and to fish (for example farmed fish).

In some embodiments, a therapeutically effective amount of the replicon RNA in a method for vaccination as described herein is at least 0.1, 0.5, 1, 2, 3, 4 or 5 µg of
15 replicon RNA per dose, and/or, at most 30, 20, 15, 10, 9, 8, 7, 6 or 5 µg per dose, such as between 0.1 and 10 µg, between 0.5 and 10 µg, between 1 and 10 µg, about 1µg, about 2.5 µg, about 5µg, about 7.5µg or about 10µg, in particular when the subject is a mammal, such as a human or domesticated animal.

20 In some embodiments, the method of vaccination comprises step (a) administration of a first dose of the replicon RNA, and, step (b) administration of a second dose of the same replicon RNA. In a further embodiment the method further comprises step (c) administration of a third dose of the same replicon RNA.

In some embodiments, the interval between step (a) and step (b) and/or the interval
25 between step (b) and step (c), is at least 4 weeks/one month, at least 6 weeks, at least 8 weeks/two months, and/or up to 18 months, up to 15 months, up to 12 months/about one year, up to 9 months, up to 6 months, up to 4 months or up to 3 months. In some embodiments, the interval between step (a) and step (b) is shorter than the interval between step (b) and step (c). The need for step (b) and/or step
30 (c) in the method of vaccination and interval between the method steps also depends on the nature of the antigen comprised in the heterologous protein expressed by the replicon RNA.

Adjuvants have been described to support (and sometimes enable) a vaccine composition comprising an antigen or antigen generating moiety to achieve a robust
35 immune response against pathogens (see Facciola A, et al. An Overview of Vaccine

Adjuvants: Current Evidence and Future Perspectives. Vaccines (Basel). 2022 May 22;10(5):819.). As illustrated in the examples, a pharmaceutical composition comprising the replicon RNA as described herein, in particular when formulated in a lipid nanoparticle as described herein, elicits a broader adaptive immune response (both humoral and cell mediated immune response) and/or raises an adaptive immune response at a lower dose than the corresponding composition comprising mRNA. The replicon RNA administered in a pharmaceutical composition as described herein thus provides for a broad immune response in the absence of any additional adjuvant. Accordingly, in some embodiments, the compositions comprising a replicon RNA as described herein for use in a method of vaccination do not comprise a further adjuvant.

The invention is further described by the following non-limiting examples which illustrate the invention and are not intended to, nor should they be interpreted to, limit the scope of the disclosure.

Examples

Example 1 - saRNA production

Plasmids are constructed encoding saRNA constructs with the following sequence from 5' to 3':

- 5' UTR (SEQ ID NO: 10) or alternative 5' UTR (SEQ ID NO: 19),
- nsPs VEEV wild-type (SEQ ID NO: 3) or containing A4488G mutation (SEQ ID NO: 4), wherein the A4488G nucleotide substitution results in an VEEV nsP3 comprising an amino acid mutation at position 167 (I167M), or codon optimized nsPs containing A4488G mutation (SEQ ID NO: 23), wherein the A4488G nucleotide substitution results in an VEEV nsP3 comprising an amino acid mutation at position 167 (I167M) or alternative VEEV nsPs (SEQ ID NO: 20), or alternative VEEV nsPs containing A4488G mutation (SEQ ID NO: 21), wherein the A4488G nucleotide substitution results in an VEEV nsP3 comprising an amino acid mutation at position 167 (I167M).
- subgenomic UTR (SEQ ID NO: 11),
- GOI selected from luciferase (SEQ ID NO: 12), eBFP (SEQ ID NO: 13), HA (SEQ ID NO: 14) or SARS-COV2 Spike (SEQ ID NO: 22)
- 3'UTR (SEQ ID NO: 15) and
- polyA tail (SEQ ID: 16) or 80A polyA tail (SEQ ID NO: 24 when indicated).

The plasmids are linearized by digestion with BspQI. The linearized plasmid is used as an IVT template with the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs (NEB)). In construct containing modified nucleotides cytosine nucleotides are

completely replaced by 5-methyl cytosine nucleotides. Next, the template DNA is digested using Turbo DNase for 30 minutes (ThermoFisher). The resulting IVT saRNA is then purified using the Monarch RNA Cleanup Kit (NEB).

The constructs used in the experiments are numbered as follows:

Construct	5'UTR	nsPs	GOI	further modification
1	SEQ ID 10	SEQ ID 3	SEQ ID 12	-
2	SEQ ID 10	SEQ ID 4	SEQ ID 12	-
3	SEQ ID 10	SEQ ID 3	SEQ ID 13	-
4	SEQ ID 10	SEQ ID 4	SEQ ID 13	-
5	SEQ ID 10	SEQ ID 3	SEQ ID 14	-
6	SEQ ID 10	SEQ ID 4	SEQ ID 14	-
7	SEQ ID 19	SEQ ID 3	SEQ ID 12	-
8	SEQ ID 19	SEQ ID 4	SEQ ID 12	-
9	SEQ ID 10	SEQ ID 20	SEQ ID 12	-
10	SEQ ID 10	SEQ ID 21	SEQ ID 12	-
11	SEQ ID 10	SEQ ID 3	SEQ ID 22	-
12	SEQ ID 10	SEQ ID 4	SEQ ID 22	-
13	SEQ ID 10	SEQ ID 4	SEQ ID 22	100% m5C
14	SEQ ID 10	SEQ ID 23	SEQ ID 22	-
15	SEQ ID 10	SEQ ID 3	SEQ ID 22	-
16	SEQ ID 10	SEQ ID 3	SEQ ID 22	m5C
17	SEQ ID 10	SEQ ID 3	SEQ ID 22	80A polyA tail
18	SEQ ID 10	SEQ ID 3	SEQ ID 25	

5

Example 2 - saRNA delivery: Lipofectamine or lipid nanoparticles (LNP)

For the purpose of testing the saRNAs, they are delivered to the cells either using Lipofectamine, or formulated in lipid nanoparticles (LNPs). Lipofectamine transfection is performed as described for example 3. LNP formulation is performed according to

the following protocol: saRNAs are complexed in LNPs using a mix of 35% ionisable cationic lipid (C12-200 or ((piperazine-1,4-diylbis(ethane-2,1-diyl))bis(azanetriyl))tetrakis(butane-4,1-diyl) tetrakis(2-hexyldecanoate) (LNPa) or ((2-(4-(2-((2-butyloctanoyl)oxy)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(butane-4,1-diyl) bis(2-butyloctanoate) (LNPb)); 20% DOPE; 1.5% DMG-PEG2000; 43.5% cholesterol using an Ignite (Precision NanoSystems). Where needed, LNPs are dialyzed overnight against buffer in Tris-HCl (10 mM, pH 7.4).

Example 3 - Bioluminescence and viability in HeLa or RD cells

10 000 HeLa cells or 25 000 rhabdomyosarcoma (RD) cells were seeded in clear bottom black wall, 96 well plates. HeLa cells were grown and seeded in MEM media (supplemented with 10% FBS, 1% Pen/strep, 1% Sodium pyruvate and 1% MEM NEAA (all Gibco)), RD cells were grown and seeded in DMEM (supplemented with 10% FBS and 1% Pen/strep). After 24 hours the cells were transfected with the saRNAs constructs expressing luciferase (constructs 1 and 2) using Lipofectamine MessengerMax (ThermoFisher) (10 ng/well for both HeLa and RD) at an RNA/lipofectamine ratio of 1:2 ($\mu\text{g}/\mu\text{l}$). The transfection happened in OptiMEM media, 4 hours after transfection the OptiMEM containing the saRNA-lipofectamine complex was removed and replaced with 50 μL growth media. At 24 hours and 48 hours after transfection, luciferase activity and viability were determined using the ONE-Glo + Tox Luciferase Reporter and Cell Viability Assay (Promega). Constructs were tested against "mock" treatment, i.e. same protocol in the absence of RNA, thus in the presence of lipofectamine only.

As illustrated in Figures 1 and 2, neither luciferase expression nor cell viability is significantly affected by the I167M mutation in nsP3, compared to wild-type nsP3, in both HeLa and RD cells. Thus, the introduction of the nsP3 mutation in the replicon RNA does not significantly reduce expression of a heterologous gene of interest, nor affect cell viability, compared to wild-type nsP3 replicon RNA and to conventional mRNA.

Example 4 - Flow cytometry time course HeLa cells

50 000 HeLa cells were seeded in 24 well plates (for media composition used see protocol 0). After 24 hours the cells were transfected with saRNA constructs expressing eBFP (constructs 3 and 4) using Lipofectamine MessengerMax (ThermoFisher) (50 ng/ well) at an RNA lipofectamine ration of 1:2 ($\mu\text{g}/\mu\text{l}$) using optiMEM. Every day from day 1 to day 7 after transfection cells were detached using trypsin and washed twice with cell staining buffer (PBS + 2 % FBS + 5 mM EDTA). Cells were then measured by flow cytometry on a Cytoflex (Beckman Coulter). eBFP

signal was measured using a 450/45 BP filter in combination with an APD detector. The median fluorescence intensity (MFI) of all cells and MFI of eBFP positive cells was used as readout.

As illustrated in Figure 3, constructs 3 and 4 provide for a similar eBFP expression profile. This further supports that the mutation at amino acid position 167 of nsP3 of the replicon RNA does not significantly reduce expression of a gene of interest compared to wild-type nsP3 replicon RNA and to conventional mRNA.

Example 5 – RNAseq/innate immunity

250 000 Hela cells per well were seeded in 6 well plates. After 24 hours the cells were transfected with saRNA test samples using Lipofectamine MessengerMax (ThermoFisher) (250 ng/ well) for transfection at a RNA:lipofectamine ratio of 1:2. At 24 hours after transfection. Total RNA was isolated using the rNeasy micro kit (QIAGEN). RNA was sequenced using the QuantSeq 3' mRNA FWD kit (Lexogen) and sequenced on a Nextseq500 (Illumina). Fastq files were trimmed using Trimmomatic and read counts were determined using kallisto with the human genome (hg38). Differential expression is determined using DESeq2.

Figure 4 shows that significantly less subgenomic RNA is generated when the nsP3 is mutated at position 167 compared to wild-type nsP3. As explained elsewhere herein, high RNA levels are a key factor in the reactogenicity of RNA therapeutics, and therefore, improved safety and tolerability is expected with replicons that produce reduced subgenomic RNA.

In addition, Figure 5 illustrates the difference in expression of itemised transcripts following transfection with saRNA encoding nsP3 mutated at position 167 compared to saRNA encoding wild-type nsPs. All transcripts related to the interferon alpha pathway are shown (black dots) to indicate differences in the expression of pro-inflammatory markers of the innate immune response. Figure 5 shows that for the vast majority of IFN-alpha pathway transcripts the expression levels are lower for the nsP3 mutated saRNA compared to the wild-type saRNA. It follows that nsP3 mutated at position 167 results in a reduced innate immune response compared to wild-type nsP3.

Example 6 – *In vivo* expression

8 week old SWISS mice (Janvier Labs) were injected intramuscularly (IM) with 4 µg (injection volume of 50 µL) of saRNA formulated in LNPs comprising C12-200 as ionisable cationic lipid.

On days 0; 1; 3; 5; 7; 10; 15; 20; 25 and 30 after administration of samples comprising constructs 1 and 2, mice were subcutaneously injected with 200 µL of 15 mg/ml luciferin and after 10 minutes luciferase activity was measured using an IVIS (Perkin-Elmer).

- 5 Figure 6 illustrates the observed *in vivo* expression of the GOI of the constructs, thereby confirming the *in vitro* observation that the nsP3 mutation at position 167 does not affect the overall GOI expression profile compared to wild-type nsP3.

Example 7 - *In vivo* adaptive immunity

- 8-week-old SWISS mice (Janvier) were injected on day 0 and day 21 with 2 µg of
10 saRNA-LNP complexes. SaRNA constructs 5 and 6 comprising HA as GOI were tested. 5 µg of conventional modified mRNA-LNP complexes were used as a control. ((2-(4-(2-((2-butyloctanoyl)oxy)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(butane-4,1-diyl)bis(2-butyloctanoate) is used as ionisable cationic lipid for the formulation of the RNA containing LNPs. Six mice were included per group, with one group per construct
15 sacrificed on day 22 after the first injection and one group sacrificed on day 50 after the first injection.

IgG antibody concentrations

- On day 50 antigen specific total IgG levels in the serum were determined by ELISA. The baseline signal was determined by analyzing serum from 5 days before the first
20 injection. The ELISA plate (Anti Mouse Total IgG kit, Thermo Fisher) was coated overnight at 4°C with 100µL/well of recombinant Influenza A H1N1 (A/California/07/2009) Hemagglutinin (HA) (Sino Biologicals) at a concentration of 1 µg/mL. The next day the plate was blocked for 1 hour at 37°C while shaking at 400 RPM, using 2x assay buffer from the Anti Mouse total IgG kit (Thermo Fisher). The
25 samples were diluted 1/40 000. A standard dilution series was made from 3200 ng/µL to 0 ng/µL diluting ½ using Influenza A H1N1 HA (A/California/07/2009) specific monoclonal antibody (Thermo Fisher, Clone 02). After blocking, samples were incubated for 1 hour at 37°C while shaking at 400 RPM. The detection antibody (Anti Mouse Total IgG kit) was added in a concentration of 0.5x and incubated for 1 hour
30 at 37°C while shaking at 400 RPM. In between every step the plate was washed between 3 – 5 times with PBS + 0.05% Tween20 using the Hydrospeed plate washer (Tecan). 100 µL of TMB was added to every well and incubated in the dark at RT for 15 min, the reaction is stopped by adding 100 µL H2SO4 1M. Read out of the plate was done at 450 nm and 560 nm after first shaking for 10 sec using the Infinite F
35 plex (Tecan).

Figure 7 shows similar anti-HA IgG responses for the saRNA encoding nsP3 with the 167 mutation compared to saRNA encoding WT nsPs and compared to conventional mRNA. It follows that saRNA encoding modified nsP3 mutated at position 167 is capable of promoting a good humoral response (e.g. post vaccination).

5 Splenocyte stimulation

Cryopreserved splenocytes were thawed and resuspended in RPMI supplemented with 10% FBS, 1% Pen/strep, 50 μ M Betamercaptoethanol (all Gibco). 10^6 cells were seeded in a round-bottom 96 well plate and rested overnight at 37°C. Splenocytes were then stimulated or not with 0.6 nmol/mL HA peptide pools (Miltenyi). Two hours after stimulation, cells were treated with transport inhibitor cocktails (eBioscience) and 6 hours after stimulation, cells were washed in CSB, and dead cells stained using the FVS620 viability stain (BD). Next, cells were stained with antibodies for 20 min at 4°C in BD Horizon™ Brilliant Stain Buffer (BD) against the following markers: CD8a (APC-H7, BD). Cells were subsequently washed 2 times with CSB & fixed (Inside Fix; Miltenyi) and permeabilized (Inside Perm; Miltenyi) according to the manufacturer's recommendations. Next, intracellular cytokines were stained with antibodies for 20 min at 4°C in BD Horizon™ Brilliant Stain Buffer Plus (BD) and Inside Perm (Miltenyi) against the following markers: CD3 (eFluor450; invitrogen), CD4 (BV605; BioLegend), IL2 (R718; BD), IFN γ (BV786; BD), TNF α (PE; BD), IL17A (AF488; BD), IL4 (PE-Cy7; Invitrogen) and IL10 (BV510; BD). Cells were subsequently washed 2 times in CSB and analyzed using the Cytoflex flowcytometer (Beckman Coulter).

Figures 8 and 9 show the generation of both CD8+ as well as CD4+ cytokine expressing T-cells upon transfection with both the saRNA constructs, whereas conventional mRNA fails to induce a robust CD8+ T-cell response. It follows that transfection with saRNA encoding modified nsP3 mutated at position 167 is capable of generating a robust cellular mediated immune response, including CD8+ T-cell response (e.g. post vaccination).

Systemic innate immune responses

On day one and day 22 after saRNA-LNP injection serum was collected to determine serum cytokine levels of IFN- γ , CXCL-1, TNF- α , CCL2, IL-12, CCL5, IL-1 β , CXCL10, GM-CSF, IL-10, IFN- β , IFN- α and IL-6 using a murine multiplex ELISA anti-virus response panel (Biolegend). Log2 of fold changes of median cytokine concentrations were calculated relative to construct 5.

Figure 10 illustrates the overall favorable trend towards less reactogenicity, shown by lower serum cytokine level measured for the saRNA encoding I167M mutated nsP3

compared to saRNA encoding WT nsPs and conventional mRNA. Cytokines with baseline measurements are not shown.

Cytokine expression in the lymph nodes and site of injection local innate immune responses

5 On day 22 after saRNA-LNP injection muscle and lymph nodes were collected for RNA isolation. qPCR was performed using the RT² Profiler PCR Arrays (QIAGEN) by QIAGEN genomic services. PPM03543A and PPM03594C were used to quantify Ifna2 and Ifnb1 transcripts respectively. PPM35281A, PPM34664A and PPM03559F were used as normalizers. Log2 of fold changes of were calculated relative to empty LNP
10 injected mice (Mock).

Figure 11 shows lower interferon alpha and beta transcripts in both lymph node and muscle tissue for the I167M mutated saRNA construct compared to wild-type saRNA.

Example 8 – moDC transfection

Monocytes were isolated by collecting the CD14⁺ positive fraction of human PBMCs
15 using MACS. Monocytes were differentiated for 5 days by stimulation with 50 ng/mL IL4 and 50 ng/mL GM-CSF. On the 5th day moDCs were transfected using Lipofectamine MessengerMax (ThermoFisher) (500 ng/well) at an RNA:Lipofectamine ratio of 1:1. 24 hours after transfection cells were measured by flow cytometry on a Cytoflex (Beckman Coulter). eBFP signal was measured using a 450/45 BP filter in
20 combination with an APD detector. The median fluorescence intensity (MFI) of all cells and MFI of eBFP positive cells was used as readout.

Figure 13 shows that a construct containing the nsP3 mutation (construct 4) is transfected in a higher percentage of human moDCs compared to the parental construct (construct 1). Figure 14 shows that the mean fluorescence of moDCs
25 transfected with construct 4 is also higher than that of construct 4.

Example 9 – Innate immunity combination constructs

50 000 Hela cells per well were seeded in 24 well plates. After 24 hours the cells were mock transfected or transfected with saRNA test samples using Lipofectamine MessengerMax (ThermoFisher) (50 ng/ well) for transfection at a RNA:lipofectamine
30 ratio of 1:2. Cells were transfected with 3 parental saRNAs (constructs 1, 7 and 9) and 3 constructs in which the A4488G mutation is introduced in the parental saRNA (constructs (2, 8 and 10). At 24 hours after transfection. Total RNA was isolated using Monarch Total RNA Miniprep kit. Transcript levels are quantified using the Luna Universal One-Step RT-qPCR Kit. NAA35 primers (F: CTCGTGTGTTACTGACAGTGC,
35 R: TGGTTCACAAGGGGTTCAAAA) and CWC15 (F: AAAAGCCACGGTTAGACCAGA, R:

GGGTTTCCGCTCAGAATGTTT) primers are used to normalise expression levels. IFNB1 primers (F: GTCAGTGTGCCTGGACCATAG, R: GTTTCGGAGGTAACCTGTAAGTC) OAS1 primers (F: TGTCCAAGGTGGTAAAGGGTG, R: CCGGCGATTAACTGATCCTG) and PKR primers (F: ACGCTTTGGGGCTAATTCTTG, R: CCCGTAGGTCTGTGAAAACTT) were used to quantify the innate response of the transfected cells.

Figures 15-17 show that when the nsP3 is mutated at position 167 compared to wild-type nsP3, similar levels of interferon signalling occur in all three constructs containing the nsP3 mutation.

Example 10 - *In vivo* adaptive immunity in mice

8-week-old SWISS mice (Janvier) were injected on day 0 and day 28 with 3 µg of saRNA-LNP complexes. SaRNA constructs 11, 12, 13 and 14 comprising SARS-COV2 Spike as GOI were tested. The saRNA constructs were formulated using or ((piperazine-1,4-diylbis(ethane-2,1-diyl))bis(azanetriyl))tetrakis(butane-4,1-diyl) tetrakis(2-hexyldecanoate) as ionisable cationic lipid for the formulation of the RNA containing LNPs. Six mice were included per group.

IgG antibody concentrations

On day 42 antigen specific total IgG levels in the serum were determined by ELISA (BM501-1.0, BioMARIC). The baseline signal was determined by analyzing serum from 5 days before the first injection.

Figure 18 shows similar anti-Spike IgG responses for the saRNA encoding nsP3 with the 167 mutation compared to saRNA encoding WT nsPs. Constructs containing the m5C nucleotide modification or codon optimized nsPs also show similar IgG responses. It follows that saRNA encoding modified nsP3 mutated at position 167 is capable of promoting a good humoral response (e.g. post vaccination).

Splenocyte stimulation

Cryopreserved splenocytes were thawed and resuspended in RPMI supplemented with 10% FBS, 1% Pen/strep, 50 µM Betamercaptoethanol (all Gibco). 10⁶ cells were seeded in a round-bottom 96 well plate and rested overnight at 37°C. Splenocytes were then stimulated or not with Spike peptide pools (Miltenyi). Two hours after stimulation, cells were treated with transport inhibitor cocktails (eBioscience) and 6 hours after stimulation, cells were washed in CSB, and dead cells stained using the FVS620 viability stain (BD). Next, cells were stained with antibodies for 20 min. at 4°C in BD Horizon™ Brilliant Stain Buffer (BD) against the following markers: CD8a (APC-H7, BD). Cells were subsequently washed 2 times with CSB & fixed (Inside Fix; Miltenyi) and permeabilized (Inside Perm; Miltenyi) according to the manufacturer's

recommendations. Next, intracellular cytokines were stained with antibodies for 20 min at 4°C in BD Horizon™ Brilliant Stain Buffer Plus (BD) and Inside Perm (Miltenyi) against the following markers: CD3 (eFluor450; invitrogen), CD4 (BV605; BioLegend), IL2 (R718; BD), IFN γ (BV786; BD), TNF α (PE; BD), IL17A (AF488; BD),
5 IL4 (PE-Cy7; Invitrogen) and IL10 (BV510; BD). Cells were subsequently washed 2 times in CSB and analyzed using the Cytoflex flowcytometer (Beckman Coulter).

Figures 19 and 20 show the generation of both CD8+ as well as CD4+ cytokine expressing T-cells upon transfection with all saRNA constructs, with constructs containing the modified nsP3 inducing similar or higher T-cell responses compared to
10 the parental construct. It follows that transfection with saRNA encoding modified nsP3 mutated at position 167 is capable of generating a robust cellular mediated immune response, including CD8+ T-cell response (e.g. post vaccination).

Systemic innate immune responses

On day one after the first saRNA-LNP injection (day 1) and on day one after the
15 second saRNA-LNP injection (day 29) serum was collected to determine serum cytokine levels of IFN- γ , CXCL-1, TNF- α , CCL2, IL-12, CCL5, IL-1 β , CXCL10, GM-CSF, IL-10, IFN- β , IFN- α and IL-6 using a murine multiplex ELISA anti-virus response panel (Biolegend). Log2 of fold changes of median cytokine concentrations were calculated relative to construct 11. Cytokines with baseline measurements were not
20 shown.

Figure 21 illustrates the overall favorable trend towards less reactogenicity, shown by lower serum cytokine level measured for the saRNA encoding I167M mutated nsP3 compared to saRNA encoding WT nsPs and conventional mRNA.

Example 11 - *In vivo* immunity in pigs

25 The present vaccination trial in pigs further demonstrates the use of replicons comprising the mutated nsP3 as described herein in vaccination: replicon RNA comprising the mutated nsP3 are compared to a commercially available mRNA construct, known under INN raxtozinameran. For proper comparison, the antigen encoding GOI of the tested replicon RNA constructs corresponds to the antigen coding
30 sequence of raxtozinameran. In addition to the nsP3 mutated replicon RNA (construct 15), further modified replicon RNA are evaluated.

A total of max. 64 (N) landrace pigs (32 females/32 immune-castrated males) of ca. 4 months of age are screened for the presence of SARS-CoV-2, PHEV, and PRCV antibodies. Animals are randomized into 8 groups (n=8) and IM vaccinated with 1000
35 μ L of drug product or 300 μ L control solution in the neck region according to a prime-

boost interval of 4 weeks. Table 1 lists the groups being tested. The negative control contained buffer only. As a reference, the commercially available mRNA vaccine raxtozinameran is used. Each of the tested saRNA constructs comprised a mutation in the nsP3 region resulting in a I167M substitution in the nsP3 protein. The saRNA of test group 4 is further modified by 100% replacement of the cytosine by 5-methyl-
5 cytosine (m5C). the saRNA of test groups 5 and 6 are further modified by providing a longer poly A tail (80A). Each of the saRNA constructs are formulated in LNPs formulated as described herein above in example 2.

The pigs are followed-up until D168, upon which moment the pigs are being
10 sacrificed. See Figure 22 for a schematic representation of the trial set-up including times of injection, blood samplings for humoral, cell mediated and innate immune response, as well as for toxicity. At sacrifice, livers, kidneys, spleen, draining lymph nodes, heart, and muscle tissue at the site of injection will be harvested for analysis as well.

15 Table 1

Group	n	saRNA	Vehicle	dosage
Negative control	4	None	Buffer	/
1	8	construct 15	LNPa	10 µg
2	8	construct 15	LNPb	10 µg
3	8	construct 16	LNPa	10 µg
4	8	construct 17	LNPa	10 µg
5	8	construct 17	LNPb	10 µg
Reference	4	raxtozinameran	Comirnaty™	30 µg

Humoral immune response read-outs

Adaptive humoral immune responses are measured on serum collected at D-7, D0, D14, D21, D28, D42, D49, D56, D70, D84, D91, D98, D112, D140, and D168. The
20 levels of total IgG in the serum is measured by ELISA. In addition, a virus neutralisation test (VNT) assay is included to quantify the neutralizing antibody titers at the same time-points as the total Ab response except for D-7.

Cellular immune response read-outs

Heparinized blood is collected at baseline D0, D28, D42, D56, D70, D84, D98, D112, D140 and D168. PBMCs are isolated to quantify adaptive cell-mediated immune
25 responses (CMI) using ELISpot and/or flow cytometry for which the following markers should be included: LD - CD3 - CD4 - CD8a - CD8b - TNFα - IL-2 - IFNγ

Innate immune response read-outs

Innate immune responses will be measured on serum collected at D0, D1, D28 and D29. One or more of the following serum cytokines will be analysed: IFN α , IFN β , IFN γ , IL-1b, IL-6, IL-10, TNF α .

5 Preliminary toxicity profile

Toxicology biomarkers will be measured in blood samples collected at various timepoints selected from D0, D28, D35, and D98, D168 to predict specific organ toxicity, with priority for liver and kidney markers. Following biomarkers can be analysed, not excluding additional markers:

- 10 • Liver: ALT and AST (less specific and less sensitive)
- Kidney: GFR, ureum, creatinine
- Muscle: Creatinine kinase (CK; less specific and less sensitive)

Histopathological analysis on the collected tissues upon sacrifice of the pigs, may be performed to further assess specific organ toxicity.

- 15 Strong humoral and cell mediated immune responses are being observed following both prime and booster administration in each of the replicon RNA treatment groups, thereby demonstrating that the alphaviral nsP3 modification can be used successfully in replicon RNA for generating the desired immune response against the antigen encoded by the GOI. The example further illustrates that the nsP3 modification
- 20 described herein can be used in combination with other modifications to the replicon RNA (*in casu*, elongation of the polyA tail and use of modified nucleosides).

Example 12 – *In vivo* immunity in pigs

- In light of the strong humoral and cell mediated immune responses being observed in example 11, a follow-up trial in pigs (7 weeks old) is being initiated using
- 25 alternative doses of the replicon RNA constructs. The replicon treatment groups dosed at 1, 5 and 10 μ g of construct 15 are being compared to the registered mRNA based vaccine Comirnaty™. In an additional replicon treatment group, a *Chlamydia trachomatis* MOMP encoding replicon (construct 18) is being administered in a 5 μ g dose. The treatment schedule provides for a prime vaccination at D0 followed by a
 - 30 booster at D28. Blood samples are taken at base line, after prime and after booster, to assess and characterise the humoral, cell mediated and innate immune responses, as well as to gather data on the toxicity profile (including reactogenicity and/or innate immune response).

Example 13 – *In vivo* immunity in non-human primates

The objective of the study is to validate the use of the replicon RNA in non-human primates and thereby bridging the data with rodents and pigs, e.g. as described herein above. To that end replicon treatment groups will receive a IM prime and boost administration of LNP formulated construct 15, either at 10 µg or 5 µg per dose with a dosing interval of 2 months/56 days. The registered mRNA based vaccine Comirnaty™ will be used as positive reference. The animals will be monitored for physical wellness parameters (e.g. inspection of site of injection, measurement of body temperature, etc.) as well as blood parameters (humoral immunity (including VNT), CMI, innate immunity and toxicity) demonstrating its use for vaccination purpose against the antigen encoded by the GOI comprised in a replicon RNA as described herein.

SEQUENCES LISTING

nsP3 protein	
SEQ ID NO:1	AA sequence of wild-type VEEV nsP3
APSYHVVVRGDIATATEGVIINAANSKGQPGGGVCGALYKKFPESFDLQPIEVGKARLVKGA AKHIIHAVGPNFNKVSEVEGDKQLAEAYESIAKIVNDNNYKSVAIPLLSTGIFSGNKDRLTQ SLNHLLTALDTTDADVAIYCRDKKWEMTLKEAVARREAVEEICISDDSSVTEPDAELVRVHP KSSLAGRKGYSTSDGKTFSYLEGTFKHQAAKDIAEINAMWPVATEANEQVCMYILGESMS SIRSKCPVEESEASTPPSTLPCLCIHAMTPERVQRLKASRPEQITVCSSFPLPKYRITGVQKI QCSQPILFSPKVPAYIHPRKYLVTETPPVDETPEPSAENQSTEGTPEQPPLITEDETRTRTPEPII IEEEEEDSISLLSDGPTHQVLQVEADIHGPPSVSSSSWSIPHASDFDVSLSILDITLEGASV TSGATSAETNSYFAKSMEFLARPVPAPRTVFRNPPHPAPRTRTPSLAPSRACSRSTSLVSTPP GVNRVITREELEALTPSRTPSRSVSRTSLVSNPPGVNRVITREEFEAFVAQQQ	
SEQ ID NO:2	Artificial sequence: AA sequence of the modified VEEV nsP3 with I167M mutation
APSYHVVVRGDIATATEGVIINAANSKGQPGGGVCGALYKKFPESFDLQPIEVGKARLVKGA AKHIIHAVGPNFNKVSEVEGDKQLAEAYESIAKIVNDNNYKSVAIPLLSTGIFSGNKDRLTQ SLNHLLTALDTTDADVAIYCRDKKWEMTLKEAVARREAVEEICMSDDSSVTEPDAELVRVH PKSSLAGRKGYSTSDGKTFSYLEGTFKHQAAKDIAEINAMWPVATEANEQVCMYILGESMS SIRSKCPVEESEASTPPSTLPCLCIHAMTPERVQRLKASRPEQITVCSSFPLPKYRITGVQKI QCSQPILFSPKVPAYIHPRKYLVTETPPVDETPEPSAENQSTEGTPEQPPLITEDETRTRTPEPII IEEEEEDSISLLSDGPTHQVLQVEADIHGPPSVSSSSWSIPHASDFDVSLSILDITLEGASV TSGATSAETNSYFAKSMEFLARPVPAPRTVFRNPPHPAPRTRTPSLAPSRACSRSTSLVSTPP GVNRVITREELEALTPSRTPSRSVSRTSLVSNPPGVNRVITREEFEAFVAQQQ	
Replicon RNA (as used in Examples)	
SEQ ID NO:3	RNA sequence coding for wild-type VEEV nsPs 1-4
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SEQ ID NO:4

Artificial sequence - RNA sequence coding for
 VEEV nsPs 1-4 with the A4488G mutation

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TATAAATACCATCACTATCAGCAGTGTGAAGACCATGCCATTAAAGCTTAGCATGTTGACC
AAGAAAGCTTGTCTGCATCTGAATCCCGGCGGAACCTGTGTGAGCATAGGTTATGGTTA
CGCTGACAGGGCCAGCGAAAGCATCATTGGTGCTATAGCGCGGCAGTTCAAGTTTCCC
GGGTATGCAAACCGAAATCCTCACTTGAAGAGACGGAAGTTCTGTTTGTATTATTGGG
TACGATCGCAAGGCCCGTACGCACAATCCTTACAAGCTTTCATCAACCTTGACCAACATT
TATACAGGTTCCAGACTCCACGAAGCCGGATGTGCACCCTCATATCATGTGGTGCGAGG
GGATATTGCCACGGCCACCGAAGGAGTGATTATAAATGCTGCTAACAGCAAAGGACAAC
CTGGCGGAGGGGTGTGCGGAGCGCTGTATAAGAAATCCCGGAAAGCTTCGATTTACA
GCCGATCGAAGTAGGAAAAGCGCGACTGTCAAAGGTGCAGCTAAACATATCATTCTAG
CCGTAGGACCAAACTTCAACAAAGTTTCGGAGGTTGAAGGTGACAAACAGTTGGCAGAG
GCTTATGAGTCCATCGCTAAGATTGTCAACGATAACAATTACAAGTCAGTAGCGATTCCA
CTGTTGTCCACCGGCATCTTTCCGGGAACAAAGATCGACTAACCCAATCATTGAACCAT
TTGCTGACAGCTTTAGACACCACTGATGCAGATGTAGCCATATACTGCAGGGACAAGAA
ATGGGAAATGACTCTCAAGGAAGCAGTGGCTAGGAGAGAAGCAGTGGAGGAGATATGC
ATGTCGCGACGACTCTTCAGTGACAGAACCTGATGCAGAGCTGGTGAGGGTGCATCCGAA
GAGTTCTTTGGCTGGAAGGAAGGGCTACAGCACAAGCGATGGCAAACCTTTCTCATATT
TGGAAGGGACCAAGTTTCACCAGGCGGCCAAGGATATAGCAGAAATTAATGCCATGTG
GCCCCGCTTGTATCTAGCTCATCCTGGTCCATTCTCATGCATCCGACTTTGATGTGG
ACAGTTTATCCATACTTGACACCCTGGAGGGAGCTAGCGTGACCAAGCGGGCAACGTCA
GCCGAGACTAACTCTTACTTCGCAAAGAGTATGGAGTTTCTGGCGCGACCGGTGCCTGC
GCCTCGAACAGTATTAGGAACCCCTCCACATCCCGCTCCGCGCACAAGAACACCGTCAC
TTGCACCCAGCAGGGCCTGCTCGAGAACCAGCCTAGTTTCCACCCCGCCAGGCGTGAAT
AGGGTGATCACTAGAGAGGAGCTCGAGGCGCTTACCCCGTCACGCACTCCTAGCAGGT
CGGTCTCGAGAACCAGCCTGGTCTCCAACCCGCCAGGCGTAAATAGGGTGATTACAAGA
GAGGAGTTTGAGGCGTTCGTAGCACAACAACAATGACGGTTTGATGCGGGTGCATACAT
CTTTTCTCCGACACCGGTCAAGGGCATTTACAACAAAAATCAGTAAGGCAAACGGTGC
TATCCGAAGTGGTGTGGAGAGGACCGAATTGGAGATTTCGTATGCCCCGCGCTCGAC
CAAGAAAAAGAAATTAATACGCAAGAAATTACAGTTAAATCCACACCTGCTAACAGA
AGCAGATACCAGTCCAGGAAGGTGGAGAACATGAAAGCCATAACAGCTAGACGTATTCT
GCAAGGCCTAGGGCATTATTTGAAGGCAGAAGGAAAAGTGGAGTGCTACCGAACCCCTG

CATCCTGTTCTTTGTATTTCATCTAGTGTGAACCGTGCCTTTTCAAGCCCCAAGGTCGCA GTGGAAGCCTGTAACGCCATGTTGAAAGAGAACTTTCCGACTGTGGCTTCTTACTGTATT ATTCCAGAGTACGATGCCTATTTGGACATGGTTGACGGAGCTTCATGCTGCTTAGACACT GCCAGTTTTTGGCCTGCAAAGCTGCGCAGCTTTCCAAAGAAACACTCCTATTTGGAACCC ACAATACGATCGGCAGTGCCTTCAGCGATCCAGAACACGCTCCAGAACGTCCTGGCAGC TGCCACAAAAAGAAATTGCAATGTCACGCAAATGAGAGAATTGCCCGTATTGGATTCCG CGGCCTTTAATGTGGAATGCTTCAAGAAATATGCGTGTAAATGAATATTGGGAAACGT TTAAAGAAAACCCCATCAGGCTTACTGAAGAAAACGTGGTAAATTACATTACCAAATTA AAGGACCAAAAAGCTGCTGCTCTTTTTGCGAAGACACATAATTTGAATATGTTGCAGGACA TACCAATGGACAGGTTTGTAATGGACTTAAAGAGAGACGTGAAAGTGAAGTCCAGGAACA AAACATACTGAAGAACGGCCCAAGGTACAGGTGATCCAGGCTGCCGATCCGCTAGCAA CAGCGTATCTGTGCGGAATCCACCGAGAGCTGGTTAGGAGATTAAATGCGGTCTGCTT CCGAACATTTCATACACTGTTTGATATGTCGGCTGAAGACTTTGACGCTATTATAGCCGAG CACTTCCAGCCTGGGGATTGTGTTCTGGAAACTGACATCGCGTCGTTTGATAAAAGTGA GGACGACGCCATGGCTCTGACCGCGTTAATGATTCTGGAAGACTTAGGTGTGGACGCA GAGCTGTTGACGCTGATTGAGCGGCTTTTCGGCGAAAATTCATCAATACATTTGCCACT AAAACATAAATTTAAATTCGGAGCCATGATGAAATCTGGAATGTTCTCACACTGTTTGTG AACACAGTCAATTAACATTGTAATCGCAAGCAGAGTGTTGAGAGAACGGCTAACCGGATC ACCATGTGCAGCATTTCATTGGAGATGACAATATCGTGAAAGGAGTCAAATCGGACAAAT TAATGGCAGACAGGTGCGCCACCTGGTTGAATATGGAAGTCAAGATTATAGATGCTGTG GTGGGCGAGAAAGCGCCTTATTTCTGTGGAGGGTTTATTTTGTGTGACTCCGTGACCGG CACAGCGTGCCGTGTGGCAGACCCCTAAAAAGGCTGTTTAAGCTTGGCAAACCTCTGG CAGCAGACGATGAACATGATGATGACAGGAGAAGGGCATTGCATGAAGAGTCAACACG CTGGAACCGAGTGGGTATTCTTTCAGAGCTGTGCAAGGCAGTAGAATCAAGGTATGAAA CCGTAGGAACTTCCATCATAGTTATGGCCATGACTACTCTAGCTAGCAGTGTTAAATCAT TCAGCTACCTGAGAGGGGGCCCTATAACTCTCTACGGC	
SEQ ID NO:5	RNA sequence coding for wild-type VEEV nsP1
ATGGAGAAAGTTCACGTTGACATCGAGGAAGACAGCCCATTCCTCAGAGCTTTGCAGCG GAGCTTCCCGCAGTTTGAGGTAGAAGCCAAGCAGGTCACTGATAATGACCATGCTAATG CCAGAGCGTTTTGCGATCTGGCTTCAAAACTGATCGAAACGGAGGTGGACCCATCCGAC ACGATCCTTGACATTGGAAGTGCGCCCGCCCGCAGAATGTATTCTAAGCACAAGTATCA TTGTATCTGTCCGATGAGATGTGCGGAAGATCCGGACAGATTGTATAAGTATGCAACTA AGCTGAAGAAAACTGTAAGGAAATAACTGATAAGGAATTGGACAAGAAAAATGAAGGAG CTCGCCGCCGTGATGAGCGACCCCTGACCTGGAAACTGAGACTATGTGCCTCCACGACGA CGAGTCGTGTGCTACGAAGGGCAAGTCGCTGTTTACCAGGATGTATACGCGGTTGAC GGACCGACAAGTCTCTATCACCAGCCAATAAGGGAGTTAGAGTCGCCCTACTGGATAGG CTTTGACACCACCCCTTTTATGTTTAAAGAACTTGGCTGGAGCATATCCATCATACTCTACC AACTGGGCCGACGAAACCGTGTTAACGGCTCGTAACATAGGCCTATGCAGCTCTGACGT TATGGAGCGGTACGTAGAGGGATGTCCATTCTTAGAAAGAAGTATTTGAAACCATCCA ACAATGTTCTATTCTCTGTTGGCTCGACCATCTACCACGAGAAGAGGGACTTACTGAGG AGCTGGCACCTGCCGTCTGTATTTCACTTACGTGGCAAGCAAAATTACACATGTCGGTGT GAGACTATAGTTAGTTGCGACGGGTACGTGTTAAAAGAATAGCTATCAGTCCAGGCCT GTATGGGAAGCCTTCAGGCTATGCTGCTACGATGCACCGCGAGGGATTCTTGCTGCTGCA AAGTGACAGACACATTGAACGGGGAGAGGGTCTCTTTTCCCGTGTGCACGTATGTGCCA GCTACATTGTGTGACCAAATGACTGGCATACTGGCAACAGATGTCAGTGCGGACGACGC GCAAAAACCTGCTGGTTGGGCTCAACCAGCGTATAGTCGTCAACGGTCGCACCCAGAGAA ACACCAATACCATGAAAAATTACCTTTTGGCCGTAGTGGCCCAGGCATTTGCTAGGTGG GCAAAGGAATATAAGGAAGATCAAGAAGATGAAAGGCCACTAGGACTACGAGATAGAC AGTTAGTCATGGGGTGTGTTGGGCTTTTGAAGGCACAAGATAACATCTATTTATAAGC GCCCCGATACCCAAACCATCATCAAAGTGAACAGCGATTTCCAATCATTCTGCTGCCC AGGATAGGCAGTAACACATTGGAGATCGGGCTGAGAACAAGAATCAGGAAAATGTTAG AGGAGCACAAAGGAGCCGTACCTCTCATTACCGCCGAGGACGTACAAGAAGCTAAGTG CGCAGCCGATGAGGCTAAGGAGGTGCGTGAAGCCGAGGAGTTGCGCGCAGCTCTACCA	

CCTTTGGCAGCTGATGTTGAGGAGCCCACTCTGGAAGCCGATGTCGACTTGATGTTACA AGAGGCTGGGGCC	
SEQ ID NO:6	RNA sequence coding for wild-type VEEV nsP2
GGCTCAGTGGAGACACCTCGTGGCTTGATAAAGGTTACCAGCTACGATGGCGAGGACA AGATCGGCTCTTACGCTGTGCTTTCTCCGAGGCTGTAAGAGTGAAAAATTATCTT GCATCCACCCTCTCGCTGAACAAGTCATAGTGATAACACACTCTGGCCGAAAAGGGCGT TATGCCGTGGAACCATACCATGGTAAAGTAGTGGTGCCAGAGGGACATGCAATACCCGT CCAGGACTTTCAAGCTCTGAGTGAAAGTGCCACCATTGTGTACAACGAACGTGAGTTCG TAAACAGGTACCTGCACCATATTGCCACACATGGAGGAGCGCTGAACACTGATGAAGAA TATTACAAAAGTGTCAAGCCCAGCGAGCACGACGGCGAATACCTGTACGACATCGACAG GAAACAGTGCCTCAAGAAAGAACTAGTCACTGGGCTAGGGCTCACAGGCGAGCTGGTG GATCCTCCCTTCCATGAATTCGCTACGAGAGTCTGAGAACACGACCAGCCGCTCCTTA CCAAGTACCAACCATAGGGGTGTATGGCGTGCCAGGATCAGGCAAGTCTGGCATCATT AAAGCGCAGTCAACAAAAAAGATCTAGTGGTGAGCGCCAAGAAAGAAAAGTGTGCAGA AATTATAAGGGACGTCAAGAAAATGAAAGGGCTGGACGTCAATGCCAGAACTGTGGACT CAGTGCTCTTGAATGGATGCAAAACACCCCGTAGAGACCCTGTATATTGACGAAGCTTTT GCTTGTCTGACGAGTACTCTCAGAGCGCTCATAGCCATTATAAGACCTAAAAAGGCAGT GCTCTGCGGGATCCCAAACAGTGCGGTTTTTTAACATGATGTGCCTGAAAGTGCATTT TAACCACGAGATTTGCACACAAGTCTTCCACAAAAGCATCTCTCGCCGTTGCACTAAATC TGTGACTTCGGTCTCTCAACCTTGTTTTACGACAAAAAATGAGAACGACGAATCCGAA AGAGACTAAGATTGTGATTGACACTACCGGCAGTACCAAACCTAAGCAGGACGATCTCA TTCTCACTTGTTCAGAGGGTGGGTGAAGCAGTTGCAAATAGATTACAAAGGCAACGAA ATAATGACGGCAGCTGCCTCTCAAGGGCTGACCCGTAAAGGTGTGTATGCCGTTCCGGTA CAAGGTGAATGAAAATCCTCTGTACGCACCCACCTCAGAACATGTGAACGTCTACTGA CCCGCACGGAGGACCGCATCGTGTGAAAAACACTAGCCGGCGACCCATGGATAAAAAAC ACTGACTGCCAAGTACCCTGGGAATTTCACTGCCACGATAGAGGAGTGGCAAGCAGAG CATGATGCCATCATGAGGCACATCTTGGAGAGACCGGACCCTACCGACGTCTTCCAGAA TAAGGCAAACGTGTGTTGGGCCAAGGCTTTAGTGCCGGTGCTGAAGACCGCTGGCATA GACATGACCACTGAACAATGGAACACTGTGGATTATTTTGAACGGACAAAGCTCACTC AGCAGAGATAGTATTGAACCAACTATGCGTGAGGTTCTTTGGACTCGATCTGGACTCCG GTCTATTTTCTGCACCCACTGTTCCGTTATCCATTAGGAATAATCACTGGGATAACTCCC CGTCGCCTAACATGTACGGGCTGAATAAAGAAGTGGTCCGTCAGCTCTCTCGCAGGTAC CCACAACCTGCCTCGGGCAGTTGCCACTGGAAGAGTCTATGACATGAACACTGGTACACT GCGCAATTATGATCCGCGCATAAACCTAGTACCTGTAAACAGAAGACTGCCTCATGCTTT AGTCCTCCACCATAATGAACACCCACAGAGTGACTTTTCTTTCATTGTCAGCAAAATTGAA GGCAGAACTGTCCTGGTGGTGGGGAAAAAGTTGTCCGTCCAGGCAAAATGTTGAC TGGTTGTCAGACCGGCTGAGGCTACCTTCAGAGCTCGGCTGGATTTAGGCATCCCAGG TGATGTGCCCAAATATGACATAATATTTGTTAATGTGAGGACCCCATATAAATACCATCA CTATCAGCAGTGTGAAGACCATGCCATTAAGCTTAGCATGTTGACCAAGAAAGCTTGTCT GCATCTGAATCCCGGCGGAACCTGTGTGAGCATAGGTTATGGTTACGCTGACAGGGCCA GCGAAAGCATCATTGGTGCTATAGCGCGGCAGTTCAAGTTTTCCCGGGTATGCAAACCG AAATCCTCACTTGAAGAGACGGAAGTTCTGTTTGTATTCAATGGGTACGATCGCAAGGC CCGTACGCACAATCCTTACAAGCTTTCATCAACCTTGACCAACATTTATACAGGTTCCAG ACTCCACGAAGCCGGATGT	
SEQ ID NO:7	RNA sequence coding for wild-type VEEV nsP3
GCACCTCATATCATGTGGTGCGAGGGGATATTGCCACGGCCACCGAAGGAGTGATTAT AAATGCTGCTAACAGCAAAGGACAACCTGGCGGAGGGGTGTGCGGAGCGCTGTATAAG AAATTCGGGAAAGCTTCGATTTACAGCCGATCGAAGTAGGAAAAGCGCGACTGGTCAA AGGTGCAGCTAAACATATCATTGATGCCGTAGGACCAAACCTTCAACAAAGTTTCGGAGG TTGAAGGTGACAAACAGTTGGCAGAGGCTTATGAGTCCATCGCTAAGATTGTCAACGAT AACAATTACAAGTCAGTAGCGATTCCACTGTTGTCCACCGGCATCTTTCCGGGAACAAA GATCGACTAACCAATCATTGAACCATTTGCTGACAGCTTTAGACACCACTGATGCAGAT GTAGCCATATACTGCAGGGACAAGAAATGGGAAATGACTCTCAAGGAAGCAGTGGCTA GGAGAGAAGCAGTGGAGGAGATATGCATATCCGACGACTCTTCAGTGACAGAACCTGA	

TGCAGAGCTGGTGAGGGTGCATCCGAAGAGTTCTTTGGCTGGAAGGAAGGGCTACAGC ACAAGCGATGGCAAACTTTCTCATATTTGGAAGGGACCAAGTTTCACCAGGCGGCCAA GGATATAGCAGAAATTAATGCCATGTGGCCCGTTGCAACGGAGGCCAATGAGCAGGTAT GCATGTATATCCTCGGAGAAAGCATGAGCAGTATTAGGTCGAAATGCCCCGTCGAAGAG TCGGAAGCCTCCACACCACCTAGCACGCTGCCTTGCTTGTGCATCCATGCCATGACTCC AGAAAGAGTACAGCGCCTAAAAGCCTCACGTCCAGAACAAATTACTGTGTGCTCATCCTT TCCATTGCCGAAGTATAGAATCACTGGTGTGCAGAAGATCCAATGCTCCCAGCCTATATT GTTCTCACCAGAAAGTGCCTGCGTATATTCATCCAAGGAAGTATCTCGTGGAACACCAC CGGTAGACGAGACTCCGGAGCCATCGGCAGAGAAACCAATCCACAGAGGGGACACCTGA ACAACCACCACTTATAACCGAGGATGAGACCAGGACTAGAACGCCTGAGCCGATCATCA TCGAAGAGGAAGAAGAGGATAGCATAAGTTTGCTGTCAGATGGCCCCGACCCACCAGGT GCTGCAAGTCGAGGCAGACATTCACGGGCCGCCCTCTGTATCTAGCTCATCCTGGTCCA TTCCTCATGCATCCGACTTTGATGTGGACAGTTTATCCATACTTGACACCCTGGAGGGAG CTAGCGTGACCAGCGGGGCAACGTCAGCCGAGACTAACTCTTACTTCGCAAAGAGTATG GAGTTTCTGGCGCGACCGGTGCCTGCGCCTCGAACAGTATTCAGGAACCCTCCACATCC CGCTCCGCGCACAAAGAACACCGTCACTTGACCCAGCAGGGCCTGCTCGAGAACCAGC CTAGTTTCCACCCCGCCAGGCGTGAATAGGGTGATCACTAGAGAGGAGCTCGAGGCGC TTACCCCGTCACGCACTCCTAGCAGGTCGGTCTCGAGAACCAGCCTGGTCTCCAACCCG CCAGGCGTAAATAGGGTGATTACAAGAGAGGAGTTTGAGGCGTTCGTAGCACACAACA ATGACGGTTTGATGCGGGTGCA	
SEQ ID NO:8	Artificial sequence - RNA sequence coding for modified VEEV nsP3 (I167M) with A501G mutation
GCACCTCATATCATGTGGTGCGAGGGGATATTGCCACGGCCACCGAAGGAGTGATTAT AAATGCTGCTAACAGCAAAGGACAACCTGGCGGAGGGGTGTGCGGAGCGCTGTATAAG AAATCCCGGAAAGCTTCGATTTACAGCCGATCGAAGTAGGAAAAGCGCGACTGGTCAA AGGTGCAGCTAAACATATCATTATGCGGTAGGACCAAACTTCAACAAAGTTTCGGAGG TTGAAGGTGACAAACAGTTGGCAGAGGCTTATGAGTCCATCGCTAAGATTGTCAACGAT AACAATTACAAGTCAGTAGCGATTCCACTGTTGTCCACCGGCATCTTTCCGGGAACAAA GATCGACTAACCAATCATTGAACCATTTGCTGACAGCTTTAGACACCACTGATGCAGAT GTAGCCATATACTGCAGGGACAAGAAATGGGAAATGACTCTCAAGGAAGCAGTGGCTA GGAGAGAAGCAGTGGAGGAGATATGCATGTCCGACGACTCTTCAGTGACAGAACCTGA TGCAGAGCTGGTGAGGGTGCATCCGAAGAGTTCTTTGGCTGGAAGGAAGGGCTACAGC ACAAGCGATGGCAAACTTTCTCATATTTGGAAGGGACCAAGTTTCACCAGGCGGCCAA GGATATAGCAGAAATTAATGCCATGTGGCCCGTTGCAACGGAGGCCAATGAGCAGGTAT GCATGTATATCCTCGGAGAAAGCATGAGCAGTATTAGGTCGAAATGCCCCGTCGAAGAG TCGGAAGCCTCCACACCACCTAGCACGCTGCCTTGCTTGTGCATCCATGCCATGACTCC AGAAAGAGTACAGCGCCTAAAAGCCTCACGTCCAGAACAAATTACTGTGTGCTCATCCTT TCCATTGCCGAAGTATAGAATCACTGGTGTGCAGAAGATCCAATGCTCCCAGCCTATATT GTTCTCACCAGAAAGTGCCTGCGTATATTCATCCAAGGAAGTATCTCGTGGAACACCAC CGGTAGACGAGACTCCGGAGCCATCGGCAGAGAAACCAATCCACAGAGGGGACACCTGA ACAACCACCACTTATAACCGAGGATGAGACCAGGACTAGAACGCCTGAGCCGATCATCA TCGAAGAGGAAGAAGAGGATAGCATAAGTTTGCTGTCAGATGGCCCCGACCCACCAGGT GCTGCAAGTCGAGGCAGACATTCACGGGCCGCCCTCTGTATCTAGCTCATCCTGGTCCA TTCCTCATGCATCCGACTTTGATGTGGACAGTTTATCCATACTTGACACCCTGGAGGGAG CTAGCGTGACCAGCGGGGCAACGTCAGCCGAGACTAACTCTTACTTCGCAAAGAGTATG GAGTTTCTGGCGCGACCGGTGCCTGCGCCTCGAACAGTATTCAGGAACCCTCCACATCC CGCTCCGCGCACAAAGAACACCGTCACTTGACCCAGCAGGGCCTGCTCGAGAACCAGC CTAGTTTCCACCCCGCCAGGCGTGAATAGGGTGATCACTAGAGAGGAGCTCGAGGCGC TTACCCCGTCACGCACTCCTAGCAGGTCGGTCTCGAGAACCAGCCTGGTCTCCAACCCG CCAGGCGTAAATAGGGTGATTACAAGAGAGGAGTTTGAGGCGTTCGTAGCACACAACA ATGACGGTTTGATGCGGGTGCA	
SEQ ID NO:9	RNA sequence coding for wild-type VEEV nsP4
TACATCTTTTCTCCGACACCGGTCAAGGGCATTACAACAAAAATCAGTAAGGCAAACG GTGCTATCCGAAGTGGTGTGGAGAGGACCGAATTGGAGATTTCGTATGCCCCGCGCCT	

CGACCAAGAAAAAGAAGAATTACTACGCAAGAAATTACAGTTAAATCCCACACCTGCTAA CAGAAGCAGATACCAAGTCCAGGAAGGTGGAGAACATGAAAGCCATAACAGCTAGACGT ATTCTGCAAGGCCTAGGGCATTATTTGAAGGCAGAAGGAAAAGTGGAGTGCTACCGAAC CCTGCATCCTGTTCTTTGTATTTCATCTAGTGTGAACCGTGCCTTTTCAAGCCCCAAGGT CGCAGTGGAAGCCTGTAACGCCATGTTGAAAGAGAACTTTCCGACTGTGGCTTCTTACT GTATTATTCAGAGTACGATGCCTATTTGGACATGGTTGACGGAGCTTCATGCTGCTTAG ACACTGCCAGTTTTTCCCCTGCAAAGCTGCGCAGCTTTCCAAAGAAACACTCCTATTTGG AACCCACAATACGATCGGCAGTGCCTTCAGCGATCCAGAACACGCTCCAGAACGTCCTG GCAGCTGCCACAAAAAGAAATTGCAATGTACGCAAAATGAGAGAATTGCCCGTATTGGA TTCGGCGGCCTTTAATGTGGAATGCTTCAAGAAATATGCGTGTAATAATGAATATTGGGA AACGTTTAAAGAAAACCCCATCAGGCTTACTGAAGAAAACGTGGTAAATTACATTACCAA ATTAAAAGGACCAAAAGCTGCTGCTCTTTTTCGGAAGACACATAATTTGAATATGTTGCA GGACATACCAATGGACAGGTTTGTAAATGGACTTAAAGAGAGACGTGAAAGTGACTCCAG GAACAAAACATACTGAAGAACGGCCCAAGGTACAGGTGATCCAGGCTGCCGATCCGCT AGCAACAGCGTATCTGTGCGGAATCCACCGAGAGCTGGTTAGGAGATTAATGCGGTCC TGCTTCCGAACATTACACTGTTTGATATGTCGGCTGAAGACTTTGACGCTATTATAG CCGAGCACTTCCAGCCTGGGGATTGTGTTCTGGAACTGACATCGCGTCGTTTGATAAA AGTGAGGACGACGCCATGGCTCTGACCGCTTAATGATTCTGGAAGACTTAGGTGTGGA CGCAGAGCTGTTGACGCTGATTGAGGCGGCTTTCGGCGAAATTCATCAATACATTTGC CCACTAAACTAAATTTAAATTCGGAGCCATGATGAAATCTGGAATGTTCTCACACTGT TTGTGAACACAGTCATTAACATTGTAATCGCAAGCAGAGTGTGAGAGAACGGCTAACC GGATCACCATGTGCAGCATTTCATTGGAGATGACAATATCGTGAAAGGAGTCAAATCGGA CAAATTAATGGCAGACAGGTGCGCCACCTGGTTGAATATGGAAGTCAAGATTATAGATG CTGTGGTGGGCGAGAAAGCGCCTTATTTCTGTGGAGGGTTTATTTTGTGTGACTCCGTG ACCGGCACAGCGTGCCGTGTGGCAGACCCCTAAAAAGGCTGTTTAAGCTTGGCAAAC CTCTGGCAGCAGACGATGAACATGATGACAGGAGAAGGGCATTGCATGAAGAGTC AACACGCTGGAACCGAGTGGGTATTCTTTCAGAGCTGTGCAAGGCAGTAGAATCAAGGT ATGAAACCGTAGGAACTTCCATCATAGTTATGGCCATGACTACTCTAGCTAGCAGTGTTA AATCATTAGCTACCTGAGAGGGGCCCCTATAACTCTCTACGGCTAA	
SEQ ID NO:10	5' UTR
ATGGGCGGCGCATGAGAGAAGCCCAGACCAATTACCTACCCAAA	
SEQ ID NO:11	subgenomic UTR
CCTGAATGGACTACGACATAGTCTAGTCCGCCAAGGCCACC	
SEQ ID NO:12	RNA sequence coding for luciferase
ATGGAAGATGCCAAAAACATTAAGAAGGGCCAGCGCCATTCTACCCACTCGAAGACGG GACCGCCGCGCAGCAGCTGCACAAAGCCATGAAGCGCTACGCCCTGGTGCCCGGCACC ATCGCCTTTACCGACGCACATATCGAGGTGGACATTACCTACGCCGAGTACTTCGAGAT GAGCGTTTCGGCTGGCAGAAGCTATGAAGCGCTATGGGCTGAATACAAACCATCGGATC GTGGTGTGCAGCGAGAATAGCTTGACGTTCTTCATGCCCGTGTGGGTGCCCTGTTTCAT CGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAGCTGCTGAACAGC ATGGGCATCAGCCAGCCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCT CAACGTGCAAAAGAAGCTACCGATCATACAAAGATCATCATCATGGATAGCAAGACCG ACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTTGCCACCCGGCTTCA ACGAGTACGACTTCGTGCCCCGAGAGCTTCGACCGGGACAAAACCATCGCCCTGATCATG AACAGTAGTGCGAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCTT GTGTCCGATTCAATCATGCCCGGACCCCATCTTCGGCAACCAGATCATCCCCGACACC GCTATCCTCAGCGTGGTGCCATTTACCACGGCTTCGGCATGTTACACAGCTGGGCTA CTTGATCTGCGGCTTTCGGGTCTGTCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGC GCAGCTTGCAAGACTATAAGATTCAATCTGCCCTGCTGGTGCCACACTATTTAGCTTCT TCGCTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGACAGAGATCGCCAGC GGCGGGGCGCCGCTCAGCAAGGAGGTAGGTGAGGCGGTGGCCAAACGCTTCCACCTA CCAGGCATCCGCCAGGGCTACGGCCTGACAGAAACAACCAGCGCCATTCTGATCACCC	

CCGAAGGGGACGACAAGCCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAA
GGTGGTGGACTTGGACACCGGTAAGACACTGGGTGTGAACCAGCGCGGCGAGCTGTGC
GTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAACCCCGAGGCTACAAACGCTCT
CATCGACAAGGACGGCTGGCTGCACAGCGGCGACATCGCCTACTGGGACGAGGACGA
GCACTTCTTCATCGTGGACCGGCTGAAGAGTCTGATCAAATACAAGGGCTACCAGGTAG
CCCCAGCCGAAGTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGACGCCGGGGT
CGCCGGCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGAGTCGTCGTGCTGGA
ACACGGTAAAACCATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTTACA
ACCGCCAAGAAGCTGCGCGGTGGTGTTCGTGGACGAGGTGCCTAAAGGACTGA
CCGGCAAGTTGGACGCCCGCAAGATCCGCGAGATTCTCATTAAAGCCAAGAAGGGCGG
CAAGATCGCCGTGTAA

SEQ ID NO:13

RNA sequence coding for eBFP

ATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTGCAGCTG
GACGGCGACGTAAACGGCCACAAGTTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCC
ACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCTG
GCCCACCTCGTGACCACCTGACCCACGGCGTGAGTGCTTCAGCCGCTACCCGAC
CACATGAAGCAGCAGACTTCTTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGAGC
GCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGA
GGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGATTTCAAGGAGGACGGC
AATATCCTGGGGCACAAGCTGGAGTACAATTCAACAGCCACAACGTCTATATCATGGC
CGACAAGCAGAAGAACGGCATCAAGGTGAAGTTCAGATCCGCCACAACATCGAGGAC
GGCAGCGTGAGCTCGCCGACCACTACCAGCAGAACACCCCATCGGCGACGGCCCCG
TGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAAC
GAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCG
GCATGGACGAGCTGTACAAGTAA

SEQ ID NO:14

RNA sequence coding for HA

ATGAAGGCCATCCTGGTGGTTCATGCTGTACACCTTCGCCACCGCCAACGCCGACACACT
GTGTATCGGATACCACGCCAACACAGCACCGACACCGTGGATACCGTGTGAAAAG
AACGTGACCGTGACACACAGCGTGAACCTGCTGGAAGATAAGCACAACGGCAAGCTGT
GCAAGCTGAGAGGCGTGGCACCTCTGCACCTGGGCAAGTGTAAATATCGCCGGCTGGAT
CCTGGGCAACCCTGAGTGTGAAAGCCTGAGCACAGCCAGCAGCTGGTCCTACATCGTG
GAAACCAGCAGCAGCGACAACGGCACATGCTACCCCGGCGACTTCATCGACTACGAGG
AACTGAGAGAACAGCTGAGCAGCGTGTCCAGCTTCGAGAGATTCGAGATCTTCCCAAG
ACCTCCAGCTGGCCCAACCACGATTCTAACAAGGGCGTGACAGCCGCCTGTCTCATGC
CGGCGCTAAGAGCTTCTACAAGAACCTGATCTGGCTGGTCAAGAAGGGCAACAGCTACC
CCAAGCTGAGCAAGAGCTACATCAACGACAAGGGCAAGAGGTGCTGGTCTCTGGG
CATCCACCATCCTAGCACATCTGCCGATCAGCAGAGCCTGTACCAGAACGCCGATGCCT
ATGTGTTCTGTGGGACGAGCCGTACAGCAAGAAGTTCAGCCCGAGATCGCCATCAG
ACCCAAAGTGCGGGATCAAGAGGGCCGCATGAAGTACTACTGGACCCTGGTGGAACCC
GGCGACAAGATCACATTTGAGGCCACCGGCAACCTGGTGGTGCCAGATATGCCTTCG
CCATGGAAGAAATGCCGGCAGCGGCATCATCATCAGCGACACCCCTGTGCACGACTG
CAACACCACCTGTCAGACACCTAAGGGCGCCATCAACACCAGCCTGCCTTTCCAGAACA
TTCACCCCATCACCATCGGCAAGTGCCCCAAATACGTGAAGTCCACCAAACTGCGGCTG
GCCACAGGCCTGAGAAATGTGCTAGCATCCAGAGCAGAGGCCTGTTCCGAGCCATTG
CCGGCTTTATCGAAGGCGGCTGGACAGGCATGGTGGACGGATGGTACGGATATCACCA
CCAGAACGAGCAAGGCAGCGGCTATGCCGCCGATCTGAAGTCTACCCAGAATGCCATC
GACGAGATCACCAACAAAGTGAACAGCGTGATCGAGAAGATGAACACCCAGTTCACCGC
CGTGGGAAAAGAGTTCAACCACCTGGAAAAGCGCATCGAGAACCTGAACAAGAAGGTG
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CGAGAGAACCCTGGACTACCACGACAGCAACGTGAAGAACCTGTACGAGAAAGTGCGG
AGCCAGCTGAAGAACAACGCCAAAGAGATCGGCAACGGCTGCTTCGAGTTCTACCACAA
GTGCGACAATACCTGCATGGAAAGCGTGAAGAATGGCACCTACGACTACCCTAAGTACA
GCGAGGAAGCCAAGCTGAACCGGGAAGAGATCGACGGCGTGAAGCTGGAAAGCACCC
GGATCTATCAGATCCTGGCCATCTACAGCACCGTGGCCAGTTCTCTGGTGTGGTGGTT

TCTCTGGGCGCTATCAGCTTCTGGATGTGCAGCAATGGCAGCCTGCAGTGCCGGATCTG CATCTGA	
SEQ ID NO:15	3' UTR
ATACAGCAGCAATTGGCAAGCTGCTTACATAGAACTCGCGGCGATTGGCATGCCGCCTT AAAATTTTTATTTTATTTTCTTTCTTTTCCGAATCGGATTTTGTITTTAATATTTT	
SEQ ID NO:16	polyA tail
AA	
SEQ ID NO:17	Alternative AA sequence of wild-type VEEV nsP3 when the stop codon is read through (*=stop codon)
APSYHVVVRGDIATATEGVIINAANSKGQPGGGVCGALYKKFPESFDLQPIEVGKARLVKGA AKHIIHAVGPNFNKVSEVEGDKQLAEAYESIAKIVNDNNYKSVAIPLLSTGIFSGNKDRLTQ SLNHLLTALDTTDADVAIYCRDKKWEMTLKEAVARREAVEEICISDDSSVTEPDALVRVHP KSSLAGRKGYSTSDGKTFSYLEGTKFHQAAKDIAEINAMWPVATEANEQVCMYILGESMS SIRSKCPVEESEASTPPSTLPCLCIHAMTPERVQRLKASRPEQITVCSSFPLPKYRITGVQKI QCSQPILFSPKVPAYIHPKYLVTETPPVDETPESAEQSTEGTPEQPPLITEDETRTRTPEPII IEEEEEDSISLLSDGPTHQVLQVEADIHGPPSVSSSSWSIPHASDFDVSLSILDTLEGASV TSGATSAETNSYFAKSMEFLARPVPAPRTVFRNPPHPAPRTRTPSLAPSRACSRSTSLVSTPP GVNRVITREELEALTPSRTPSRSVSRTSLVSNPPGVNRVITREEFEAFVAQQQ* RFDAGA	
SEQ ID NO:18	Alternative AA sequence of the modified VEEV nsP3 with I167M mutation when the stop codon is read through (*=stop codon)
APSYHVVVRGDIATATEGVIINAANSKGQPGGGVCGALYKKFPESFDLQPIEVGKARLVKGA AKHIIHAVGPNFNKVSEVEGDKQLAEAYESIAKIVNDNNYKSVAIPLLSTGIFSGNKDRLTQ SLNHLLTALDTTDADVAIYCRDKKWEMTLKEAVARREAVEEICMSDDSSVTEPDALVRVH PKSSLAGRKGYSTSDGKTFSYLEGTKFHQAAKDIAEINAMWPVATEANEQVCMYILGESMS SIRSKCPVEESEASTPPSTLPCLCIHAMTPERVQRLKASRPEQITVCSSFPLPKYRITGVQKI QCSQPILFSPKVPAYIHPKYLVTETPPVDETPESAEQSTEGTPEQPPLITEDETRTRTPEPII IEEEEEDSISLLSDGPTHQVLQVEADIHGPPSVSSSSWSIPHASDFDVSLSILDTLEGASV TSGATSAETNSYFAKSMEFLARPVPAPRTVFRNPPHPAPRTRTPSLAPSRACSRSTSLVSTPP GVNRVITREELEALTPSRTPSRSVSRTSLVSNPPGVNRVITREEFEAFVAQQQ* RFDAGA	
SEQ ID NO: 19	Alternative 5'UTR
ATGAGGCGGCGTCATGAGAGAAGCCAGACCAATTACCTACCCAAA	
SEQ ID NO: 20	RNA sequence coding for alternative VEEV nsPs 1- 4
ATGGAGAAAGTTCACGTTGACATCGAGGAAGACAGCCCATTCCTCAGAGCTTTGCAGCG GAGCTTCCCGCAGTTTGAGGTAGAAGCCAAGCAGGTCACTGATAATGACCATGCTAATG CCAGAGCGTTTTCGCATCTGGCTTCAAAACTGATCGAAACGGAGGTGGACCCATCCGAC ACGATCCTTGACATTGGAAGTGCGCCCGCCCGCAGAATGTATTCTAAGCACAAGTATCA TTGTATCTGTCCGATGAGATGTGCGGAAGATCCGGACAGATTGTATAAGTATGCAACTA AGCTGAAGAAAACTGTAAGGAAATAACTGATAAGGAATTGGACAAGAAAAATGAAGGAG CTCGCCGCCGTCATGAGCGACCCTGACCTGGAACTGAGACTATGTGCCTCCACGACGA CGAGTCGTGTGCTACGAAGGGCAAGTCGCTGTTTACCAGGATGTATACGCGGTTGAC GGACCGACAAGTCTCTATCACCAAGCCAATAAGGGAGTTAGAGTCGCCCTACTGGATAGG CTTTGACACCACCCCTTTTATGTTTAAGAACTTGGCTGGAGCATATCCATCATACTCTACC AACTGGGCCGACGAAACCGTGTTAACGGCTCGTAACATAGGCCTATGCAGCTCTGACGT TATGGAGCGGTACGTAGAGGGATGTCCATTCTTAGAAAGAAGTATTTGAAACCATCCA ACAATGTTCTATTCTCTGTTGGCTCGACCATCTACCACGAGAAGAGGGACTTACTGAGG AGCTGGCACCTGCCGTCTGTATTTCACTTACGTGGCAAGCAAAATTACACATGTGGTGT GAGACTATAGTTAGTTGCGACGGGTACGTCGTTAAAGAATAGCTATCAGTCCAGGCCT	

GTATGGGAAGCCTTCAGGCTATGCTGCTACGATGCACCGCGAGGGATTCTTGTGCTGCA
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GCCACCAATACCATGAAAAATTACCTTTTGGCCGTAGTGGCCCAGGCATTTGCTAGGTG
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GCGCAGCCGATGAGGCTAAGGAGGTGCGTGAAGCCGAGGAGTTGCGCGCAGCTCTAC
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ACGATGGCGAGGACAAGATCGGCTCTTACGCTGTGCTTTCTCCGAGGCTGTACTCAAG
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GAACACTGATGAAGAATATTACAAAACCTGTCAAGCCCAGCGAGCACGACGGCGAATACC
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CTAGCAACAGCGTATCTGTGCGGAATCCACCGAGAGCTGGTTAGGAGATTAAATGCGGT
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TATGAAACCGTAGGAACTTCCATCATAGTTATGGCCATGACTACTCTAGCTAGCAGTGTT AAATCATTCAGCTACCTGAGAGGGGCCCTATAACTCTCTACGGC	
SEQ ID NO: 21	Artificial sequence - RNA sequence coding for alternative VEEV nsPs 1-4 with the A4488G mutation
ATGGAGAAAGTTCACGTTGACATCGAGGAAGACAGCCCATTCTCAGAGCTTTGCAGCG GAGCTTCCCGCAGTTTGAGGTAGAAGCCAAGCAGGTCAGTATAATGACCATGCTAATG CCAGAGCGTTTTGCGATCTGGCTTCAAACTGATCGAAACGGAGGTGGACCCATCCGAC ACGATCCTTGACATTGGAAGTGCGCCCGCCCGCAGAATGTATTCTAAGCACAAAGTATCA TTGTATCTGTCCGATGAGATGTGCGGAAGATCCGGACAGATTGTATAAGTATGCAACTA AGCTGAAGAAAACTGTAAGGAAATAACTGATAAGGAATTGGACAAGAAAAATGAAGGAG CTCGCCGCCGTCATGAGCGACCCTGACCTGGAACTGAGACTATGTGCCTCCACGACGA CGAGTCGTGTGCTACGAAGGGCAAGTCGCTGTTTACCAGGATGTATACGCGGTTGAC GGACCGACAAAGTCTCTATCACCAAGCCAATAAGGGAGTTAGAGTCGCCTACTGGATAGG CTTTGACACCACCCCTTTTATGTTTAAGAACTTGGCTGGAGCATATCCATCATACTCTACC AACTGGGCGGACGAAACCGTGTTAACGGCTCGTAACATAGGCCTATGCAGCTCTGACGT TATGGAGCGGTACGTAGAGGGATGTCCATTCTTAGAAAGAAGTATTTGAAACCATCCA ACAATGTTCTATTCTCTGTTGGCTCGACCATCTACCACGAGAAGAGGGACTTACTGAGG AGCTGGCACCTGCCGTCTGTATTTCACTTACGTGGCAAGCAAAATTACACATGTCGGTGT GAGACTATAGTTAGTTGCGACGGGTACGTCGTTAAAAGAATAGCTATCAGTCCAGGCCT GTATGGGAAGCCTTCAGGCTATGCTGCTACGATGCACCGCGAGGGATTCTTGTGCTGCA AAGTGACAGACACATTGAACGGGGAGAGGGTCTCTTTTCCCGTGTGCACGTATGTGCCA GCTACATTGTGTGACCAAATGACTGGCATACTGGCAACAGATGTCAGTGCGGACGACGC GCAAAAAGTCTGGTTGGGCTCAACCAGCGTATAGTCGTCAACGGTCGCACCCAGAGA GCCACCAATACCATGAAAAATTACCTTTGCCCGTAGTGGCCAGGCATTTGCTAGGTG GGCAAAGGAATATAAGGAAGATCAAGAAGTGAAGGCCACTAGGACTAGGATAGAGA CAGTTAGTCATGGGGTGTGTTGGGCTTTTAGAAGGCACAAGATAACATCTATTTATAAG CGCCCGGATACCCAAACCATCATCAAAGTGAACAGCGATTTCCACTCATTCGTGCTGCC CAGGATAGGCAGTAACACATTGGAGATCGGGCTGAGAACAAGAATCAGGAAAATGTTA GAGGAGCACAAAGGAGCCGTCACCTCTCATTACCGCCGAGGACGTACAAGAAGCTAAGT GCGCAGCCGATGAGGCTAAGGAGGTGCGTGAAGCCGAGGAGTTGCGCGCAGCTCTAC CACCTTTGGCAGCTGATGTTGAGGAGCCCACTCTGGAAGCCGATGTCGACTTGATGTTA CAAGAGGCTGGGGCCGGCTCAGTGGAGACACCTCGTGGCTTGATAAAGGTTACCAGCT ACGATGGCGAGGACAAGATCGGCTCTTACGCTGTGCTTTCTCCGCAGGCTGTACTCAAG AGTGAAAAATTATCTTGCATCCACCCTCTCGCTGAACAAGTCATAGTGATAACACACTCT GGCCGAAAAGGGCGTTATGCCGTGGAACCATACCATGGTAAAGTAGTGGTGCCAGAGG GACATGCAATACCCGTCCAGGACTTTCAAGCTCTGAGTGAAAGTGCCACCATTGTGTAC AACGAACGTGAGTTCGTAAACAGGTACCTGCACCATATTGCCACACATGGAGGAGCGCT GAACACTGATGAAGAATATTACAAAAGTGTCAAGCCCAGCGAGCACGACGGCGAATACC TGTACGACATCGACAGGAAACAGTGCGTCAAGAAAGAACTAGTCACTGGGCTAGGGCT CACAGGCGAGCTGGTGGATCCTCCCTTCCATGAATTCGCCTACGAGAGTCTGAGAACAC GACCAGCCGCTCCTTACCAAGTACCAACCATAGGGGTGTATGGCGTGCCAGGATCAGG CAAGTCTGGCATCATTAAAAGCGCAGTCACCAAAAAAGATCTAGTGGTGAGCGCCAAGA AAGAAAAGTGTGCAGAAATTATAAGGGACGTCAAGAAAATGAAAGGCTGGAGCTCAAT GCCAGAACTGTGGACTCAGTGCTCTTGAATGGATGCAAACACCCCGTAGAGACCCTGTA TATTGACGAAGCTTTTGCTTGTATGCAGGTAATCTCAGAGCGCTCATAGCCATTATAAG ACCTAAAAGGCGAGTGCTCTGCGGGGATCCCAAACAGTGCGGTTTTTTTTAATGATGT GCCTGAAAGTGCATTTTAACCACGAGATTTGCACACAAGTCTTCCACAAAAGCATCTCTC GCCGTTGCACTAAATCTGTGACTTCGGTCTGCTCAACCTTGTTTTACGACAAAAAATGA GAACGACGAATCCGAAAGAGACTAAGATTGTGATTGACACTACCGGCAGTACCAAACCT AAGCAGGACGATCTCATTCTCACTTGTTTCAAGGGTGGGTGAAGCAGTTGCAAAATAGA TTACAAAGGCAACGAAATAATGACGGCAGCTGCCTCTCAAGGGCTGACCCGTAAAGGTG TGTACCGCTTCGGTACAAGGTGAATGAAAATCCTCTGTACGCACCCACCTCAGAACAT GTGAACGCTCTACTGACCCGACGAGGACCGCATCGTGTGGAACACACTGCCGCGC ACCCATGGATAAAAAACTGACTGCCAAGTACCCTGGGAATTTCACTGCCACGATAGAG	

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SEQ ID NO: 22

RNA sequence coding for SARS-COV2 Spike

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SEQ ID NO: 23

Artificial sequence – Codon optimized RNA
 sequence coding for VEEV nsPs 1-4 with the
 A4488G mutation

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SEQ ID NO:24	80A polyA tail
AA AAAAAAAAAAAAAAAAAAAAAAAA	
SEQ ID NO:25	RNA sequence encoding for Chlamydia trachomatis protein MOMP
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CLAIMS

1. An alphaviral non-structural protein 3 (nsP3) comprising a single amino acid mutation at amino acid position equivalent to amino acid position 167 of wild-type Venezuelan equine encephalitis virus (VEEV) nsP3 of SEQ ID NO:1.
- 5 2. An alphaviral nsP3, comprising an amino acid residue other than Ile, Leu or Val, such as a Met residue, at amino acid position equivalent to amino acid position 167 of wild-type VEEV nsP3.
3. The alphaviral nsP3 of claim 1 or 2, wherein the alphaviral nsP3 is a VEEV nsP3 comprising a single amino acid mutation at amino acid position 167, optionally a
10 I167M mutation.
4. The alphaviral nsP3 of claim 3, wherein the VEEV nsP3 has an amino acid sequence of SEQ ID NO:2 or SEQ ID NO:18.
5. A nucleic acid comprising a sequence encoding an alphaviral nsP3 of any one of claims 1-4.
- 15 6. A replicon RNA comprising a sequence encoding alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, wherein said alphaviral nsP3 is an alphaviral nsP3 of any one of claims 1-4.
7. A replicon RNA comprising a sequence encoding alphaviral non-structural proteins nsP1, nsP2, nsP3 and nsP4, said sequence modified at least by a mutation at the
20 nucleotide position equivalent to position 4488 of the sequence encoding the wild-type VEEV nsPs 1-4 of SEQ ID NO:3.
8. The replicon RNA according to claims 6 or 7, wherein the alphavirus from which the sequence encoding the nsPs1-4 are derived is selected from VEEV (such as TC-83 VEEV or Trinidad donkey virus), Everglades virus, Tonate virus, Mucambo virus
25 Cabassou virus, Mosso das pedras virus, Rio Negro virus, Pixuna virus and variants thereof.
9. The replicon RNA according to any one of claims 6-8, wherein the sequence encoding the alphaviral nsPs1-4 is derived from VEEV, or has at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%
30 sequence identity to the wild-type sequence encoding VEEV nsPs1-4 of SEQ ID NO:3.
10. The replicon RNA according to claim 9, wherein the sequence encoding the alphaviral nsPs1-4 has a nucleotide sequence of SEQ ID NO:4.
11. The replicon RNA according to any one of claims 6-10, further comprising a 5'UTR, one or more heterologous sequences encoding one or more heterologous gene

products, wherein each heterologous sequence is operably linked to one or more subgenomic promoters and/or IRES elements, and a 3'UTR.

12. The replicon RNA according to claim 11, wherein one or more of the heterologous sequences encodes a heterologous protein, such as a heterologous antigenic protein.

5 13. A method of expressing one or more heterologous gene products in a cell, comprising: introducing a replicon RNA of any one of claims 6-12 into a cell; and expressing the one or more heterologous gene products encoded by said replicon RNA in the cell.

10 14. A cell comprising a replicon RNA of any one of claims 6-12, optionally wherein the cell is a mammalian cell, such as a human cell.

15. A nanoparticle comprising a replicon RNA according to any one of claims 6-12, optionally wherein the nanoparticle is a lipid nanoparticle.

15 16. A pharmaceutical composition, comprising: the replicon RNA of any one of claims 6-12, or, nanoparticles of claim 15 and at least one carrier, optionally wherein the pharmaceutical composition comprises one or more nanoparticles comprising said replicon RNA.

17. A pharmaceutical composition comprising a cell according to claim 14 and at least one carrier.

20 18. The pharmaceutical composition of claims 16 or 17, wherein the replicon RNA encodes a heterologous protein, such as a heterologous protein for use in therapy or vaccination.

25 19. A method of vaccinating a subject, comprising the step of administering a therapeutically effective amount of the pharmaceutical composition of claim 16 comprising a replicon RNA according to claim 12 to a subject in need thereof, thereby causing expression of the heterologous protein and an immune response in the subject, optionally wherein the therapeutically effective amount is 1 to 10 µg per dose.