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(54) Title: SYNTHETIC RIG-I-LIKE RECEPTOR AGONISTS

(57) Abstract: The present disclosure relates to, *inter alia*, RNA molecules (e.g., RNA hairpin agonists) that bind to and agonize RIG-I-like receptors (RLRs), and to use of the molecules, including RLR agonists packaged in virus like particles (VLPs), in methods for treating, or ameliorating one or more symptoms of, a disorder (e.g., cancer).

# SYNTHETIC RIG-I-LIKE RECEPTOR AGONISTS

## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/925,120, filed October 23, 2019. The entire contents of which is incorporated herein by reference.

## BACKGROUND

Exogenous nucleic acids, particularly viral nucleic acids, introduced into cells induce an innate immune response, resulting in, among other events, interferon (IFN) production and cell death. Upon sensing viral RNA, RIG-I-like receptors induce type I interferon (IFN) secretion leading to upregulation of antiviral IFN-induced proteins in the infected and neighboring cells, which inhibits virus replication. Further downstream events attract immune cells and trigger the adaptive immune response. In addition, RIG-I ligands have been reported to induce the apoptosis of many different types of tumor cells, but not of normal cells.

Virus-like particles (VLPs) are supermolecular structures built in a symmetric manner from many protein molecules of one or more types. They lack the viral genome and, therefore, are noninfectious. VLPs can often be produced in large quantities by heterologous expression and can be easily be purified.

VLPs are used in the fields of vaccinology, immunology and medicine because of both their structural properties and their non-infectious nature. VLPs have been shown to be efficiently presented on MHC class I molecules as they, presumably after uptake by micropinocytosis or other cell uptake pathways, are efficiently processed and cross-primed onto MHC class I.

There remains a need for additional and improved compositions and methods to modulate the activity of immunomodulatory proteins. Such agents can be used for cancer immunotherapy and treatment of other conditions, such as chronic infection. There is a need to develop improved RIG-I-like receptor ligands, including improved delivery methods, for diverse therapeutic immunomodulatory applications.

## SUMMARY OF THE DISCLOSURE

The present disclosure is based, at least in part, on the discovery of synthetic RNA molecules that function as RIG-I-like receptor (RLR) agonists. The disclosure also provides compositions and methods for improving biological activity by packaging immunostimulatory nucleic acids, in particular RLR agonists, into VLPs (RIG-VLPs). The compositions described herein can be used to induce strong and sustained immune responses particularly useful for the treatment of tumors.

Accordingly, in some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RIG-I like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the RLR agonist comprises a ribonucleic acid (RNA) of 10-100 nucleotides in length, wherein the 5' most nucleotide of the RNA comprises a 5'diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RIG-I like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the RLR agonist comprises a ribonucleic acid (RNA) of 10-100 nucleotides in length, wherein the 5' most nucleotide of the RNA comprises a 5'diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the at least one RLR agonist is packaged in the virus-like particle.

In any of the foregoing or related aspects, the RNA is singled stranded. In other aspects, some or all of the RNA is double stranded.

In any of the foregoing or related aspects, the RNA of the RLR agonist is 10-15, 15-20, 20-25, 25-30 or 30-35 nucleotides in length.

In any of the foregoing or related aspects, the RLR agonist comprises a first polynucleotide and a second polynucleotide, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex. In some aspects, the duplex comprises a hairpin. In some aspects, the duplex comprises 10-15, 15-20, 20-25, 25-30 or 30-35 base pairs. In some aspects,

the duplex comprises less than 19 base pairs. In some aspects, the first polynucleotide is connected to the second polynucleotide by a linker.

In any of the foregoing or related aspects, the RLR agonist comprises a sequence motif that provides at least one biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif. In some aspects, the first polynucleotide comprises the sequence motif, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif. In some aspects, the first polynucleotide comprises the sequence motif, wherein the at least one RLR agonist is packaged in the virus-like particle.

In any of the foregoing or related aspects, the RLR agonist comprises a sequence motif selected from the group consisting of:

(i) a GT-repeat motif;

(ii) a GA-repeat motif;

(iii) a AUCG-repeat motif;

(iv) an AU-repeat motif;

(v) a dipyrimidine motif;

(vi) a dipurine motif;

(vii) a pyrimidine triplet motif;

(viii) a purine triplet motif;

(ix) a palindromic sequence motif; and

(x) a combination of any of (i)-(ix).

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RIG-I like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises a sequence motif selected from:

(i) a GT-repeat motif;

(ii) a GA-repeat motif;

(iii) a AUCG-repeat motif;

(iv) an AU-repeat motif;

(v) a dipyrimidine motif;

(vi) a dipurine motif;

(vii) a pyrimidine triplet motif;

(viii) a purine triplet motif;

(ix) a palindromic sequence motif; and

(x) a combination of any of (i)-(ix), wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the RLR agonists of the disclosure comprise a combination of sequence motifs. In some aspects the combination of sequence motifs is a GT-repeat motif and a purine triplet motif. In some aspects, the combination of sequence motifs is an AUCG-repeat motif and a dipyrimidine motif. In some aspects, the combination of sequence motifs is an AUCG-repeat motif and a dipurine motif.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the at least one improved biological activity is selected from:

(i) an increase in RLR-mediated cytokine production;

(ii) an increase in RLR-mediated expression of interferon-stimulated genes;

(iii) an increase in RLR-mediated intracellular signaling;

(iv) an increase in binding affinity to RLRs; and

(v) a combination of any of (i)-(iv).

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif that increases RLR-mediated type I interferon (e.g., IFN-α, IFN-β) production relative to an agonist that does not comprise the sequence motif. In some aspects, the RLR agonists of the disclosure comprise a sequence motif that increases RLR-mediated IL-1β production relative to an agonist that does not comprise the sequence motif. In some aspects, the RLR agonists of the disclosure comprise a sequence motif that increases RLR-mediated IP-10 production relative to an agonist that does not comprise the sequence motif. In some aspects, the RLR agonists of the disclosure comprise a sequence motif that increases RLR-mediated IL-6, IL-12p70, MCP-1 and/or MIP-1β production relative to an agonist that does not comprise the sequence motif.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a GT-repeat motif (e.g., GTGTGT) comprising a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 5-10, about 5, about 4, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of <19 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of about 15-18 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat

motif comprising a sequence of about 15 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of about 10-15 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of about 10 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of about 5-10 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of about 5 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of about 4 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the GT-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a GT-repeat motif comprising a sequence of 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 18 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 16 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 14 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 12 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 10 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 8 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 6 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GT-repeat motif comprising a sequence of 4 guanine and thymine nucleotides, or derivatives or analogs thereof. In some aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence

motif is a GT-repeat motif, wherein the GT-repeat motif is $[GT]_n$, wherein n = 2 to 9, 3-7, or 4-8. In some aspects, the GT-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to (operably linked to) a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a GT-repeat motif comprising a sequence of about 14 guanine and thymine nucleotides, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to (operably linked to) a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a GT-repeat motif comprising a

sequence of about 14 guanine and thymine nucleotides, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the sequence motif is a GT-repeat motif, wherein the GT-repeat motif is $[GT]_7$. In some aspects, the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a GT-repeat motif comprising a sequence of 6 guanine and thymine nucleotides, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a GT-repeat motif comprising a sequence of 6

guanine and thymine nucleotides, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the sequence motif is a GT-repeat motif, wherein the GT-repeat motif is $[GT]_3$. In some aspects, the sequence motif is a GT-repeat motif, wherein the GT-repeat motif is $[GT]_3$, and wherein the GT-repeat is followed by a purine triplet and UCG, respectively. In some aspects, the purine triplet is GGA. In some aspects, the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a GA-repeat motif (e.g., GAGAGA) comprising a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 5-10, about 5, about 4, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of <19 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 15-18 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 15 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 10-15 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 10 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 5-10 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 5 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of about 4 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the GA-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif is a GA-repeat motif comprising a sequence of 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 18 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 16 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 14 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 12 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 8 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 6 guanine and adenine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a GA-repeat motif comprising a sequence of 4 guanine and adenine nucleotides, or derivatives or analogs thereof.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a GA-repeat motif, wherein the GA-repeat motif is $[GA]_n$, where n = 2 to 9, 3 to 7 or 4 to 8. In some aspects, the GA-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, the disclosure provides composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an

agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a GA-repeat motif comprising a sequence of about 14 guanine and adenine nucleotides, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a GA-repeat motif comprising a sequence of about 14 guanine and adenine nucleotides, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the sequence motif is a GA-repeat motif, wherein the GA-repeat motif is $[GA]_7$. In some aspects, the GA-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a AUCG-repeat motif (e.g., AUCGAUCG) comprising a sequence of <19, about 16, about 12-16, about 12, about 8-12, about 6, 16, 12, 8 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of <19 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of about 16 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a

AUCG-repeat motif comprising a sequence of about 12-16 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of about 12 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of about 8-12 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of about 6 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of 16 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of 12 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the sequence motif is a AUCG-repeat motif comprising a sequence of 8 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof. In some aspects, the AUCG-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_n$, where n = 2 to 4 or 2, 3 or 4. In some aspects, the AUCG-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or

triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a AUCG-repeat motif comprising a sequence of about 12 guanine and adenine nucleotides. In some aspects, the AUCG-repeat motif is $[AUCG]_3$, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to an RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, wherein the first polynucleotide comprises the sequence motif, and wherein the sequence motif is a AUCG-repeat motif comprising a sequence of about 12 guanine and adenine nucleotides. In some aspects, the AUCG-repeat motif is $[AUCG]_3$, wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the AUCG-repeat motif provides an improved biological activity in the RLR agonist, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a AUCG-repeat motif, wherein the motif is preceded by a CG or a dipyrimidine motif. In some aspects, the AUCG-repeat motif is preceded by a CG. In some aspects, the AUCG-repeat motif is $[AUCG]_3$ and is preceded by a CG. In some aspects, the AUCG-repeat motif is $[AUCG]_3$ and is preceded by the dipyrimidine motif CC.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein the motif is preceded by a dipurine motif. In some aspects, the

dipurine motif is GA. In some aspects, the AUCG-repeat motif is $[AUCG]_3$ and is preceded by the dipurine motif GA. In some aspects, the AUCG-repeat motif is preceded by the dipurine motif II.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein one or more uridine nucleosides (U) are substituted with a modified nucleoside. In some aspects, wherein the modified nucleoside is ribothymidine (T). In some aspects, the AUGC-repeat motif is $[AUCG]_3$, wherein the one or more uridine nucleosides (U) comprising the AUCG-repeat motif are substituted with a modified nucleoside, wherein the modified nucleoside is ribothymidine (T). In some aspects, the AUGC-repeat motif is $[AUCG]_3$, wherein the one or more uridine nucleosides (U) comprising the AUCG-repeat motif are substituted with a modified nucleoside, wherein the modified nucleoside is ribothymidine (T), and wherein the AUGC-repeat motif is preceded by GG.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein one or more guanosine nucleosides (G) are substituted with a modified nucleoside. In some aspects, the modified nucleoside is inosine (I). In some aspects, the AUGC-repeat motif is $[AUCG]_3$, wherein the one or more guanosine nucleosides (G) comprising the AUCG-repeat motif are substituted with a modified nucleoside, wherein the modified nucleoside is ribothymidine (T), and wherein the AUGC-repeat motif is preceded by GG.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a AUCG-repeat motif, wherein the motif is preceded by a IG. In some aspects, the AUCG-repeat motif is $[AUCG]_3$ and is preceded by a IG.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise an AUCG-repeat, wherein one or more guanosine nucleosides (G) are substituted with an inosine (I), wherein the AUCG-repeat is preceded by an inosine (I). In some aspects, the guanosine nucleosides (G) comprising the AUCG-repeat are substituted with an inosine (I), wherein the AUCG-repeat is preceded by an inosine (I), wherein the 5' most nucleotide of the first polynucleotide comprises inosine (I).

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$. In some aspects, the sequence motif is an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$, and wherein the AUCG-repeat motif is preceded by a dipurine motif. In some aspects, the sequence motif is an

AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$, wherein the AUCG-repeat motif is preceded by a dipurine motif, and wherein the dipurine motif is GG.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$, and wherein the AUCG-repeat motif is preceded by a purine triplet motif. In some aspects, the purine triplet motif is GGG. In some aspects, the sequence motif is an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$, wherein the AUCG-repeat motif is preceded by a purine triplet motif, and wherein the purine triplet motif is GGG. In some aspects, the sequence motif is an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$, and wherein the AUCG-repeat motif is preceded by CCCCCG. In some aspects, the sequence motif is an AUCG-repeat motif, wherein the AUCG-repeat motif is $[AUCG]_2$, and wherein the AUCG-repeat motif is preceded by TCGUCG.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof; wherein the agonist comprises a [AUCG]n repeat motif, where n=2-4; wherein the 5' most AUCG repeat motif is preceded by GG, CG, or IG, and wherein the at least one RLR agonist is packaged in the virus-like particle. In some aspects, n=3. In some aspects, each G in the AUCG motif is substituted by inosine.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof; wherein the agonist comprises a [AUCG]n repeat motif, where n=2-4; wherein the 5' most AUCG repeat motif is preceded by GG, CG, or

IG, and wherein the at least one RLR agonist is packaged in the virus-like particle. In some aspects, n=3. In some aspects, each G in the AUCG motif is substituted by inosine.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a palindromic sequence comprising a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of <19 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of about 15-18 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of about 15 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of about 10-15 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of about 10 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 18 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence 17 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 16 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 15 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 14 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 13 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 12 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 11

nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 10 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 9 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 8 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 7 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 6 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 5 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome. In some aspects, the sequence motif is a palindromic sequence comprising a sequence of 4 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise a linker, wherein the linker is flanked by AU. In some aspects, the linker is flanked by an AU-repeat motif, wherein the AU-repeat motif is $[AU]_n$, where n = 2 to 3. In some aspects, the AU-repeat motif is $[AU]_2$.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein at least one of N1, N2, N3, and N4 is inosine and/or at least one of X1 and/or X2 comprises at least one inosine nucleoside, and wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(iv) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(v) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(vi) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein at least one of N1, N2, N3, and N4 is inosine and/or at least one of X1 and/or X2 comprises at least one inosine nucleoside, and wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the RLR agonist of the disclosure has an improved biological activity, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein inosine, if present, base pairs with cytidine,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein inosine, if present, base pairs with cytidine,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the RLR agonist of the disclosure has an improved biological activity, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, N1 comprises inosine and N4 comprises cytidine. In some aspects, N1 comprises inosine and N4 comprises cytidine and X1 and X2 are each 12 nucleotides in length. In some aspects, N1 comprises cytidine and N4 comprises inosine. In some aspects, N2 comprise inosine and N3 comprises cytidine. In some aspects, N2 comprises cytidine and N3 comprises inosine. In some aspects, N1 comprises guanosine. In some aspects, N2 comprises guanosine. In some aspects, N1 comprises cytidine. In some aspects, N2 comprises cytidine. In some aspects, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine. In some aspects, N1 and N2 comprise cytidine and N3 and N4 comprise guanosine. In some aspects, N1 and N2 comprise inosine and N3 and N4 comprise cytidine. In some aspects, N1 and N2 comprise cytidine and N3 and N4 comprise inosine.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise the formula:

5’-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3’, wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein inosine, if present, base pairs with cytidine, and wherein N1 comprises inosine and N4 comprises cytidine, and X1 and/or X2 each comprise at least one inosine. In some aspects, N2 comprises inosine and N3 comprises cytidine, and X1 and/or X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise guanosine N3 and N4 comprise cytidine, and X1 and/or X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and X1 and X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, X1 and X2 each comprise at least one inosine, and X1 and X2 are each 12 nucleotides in length. In some aspects, N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and X1 and X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and X1 and X2 each comprise inosine and no guanosine nucleosides. In some aspects, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, X1 and X2 each comprise at least one inosine, and X1 and X2 are each 12 nucleotides in length. In some aspects, N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and X1 and X2 each comprise inosine and no guanosine nucleosides. In some aspects, the RLR agonist of the disclosure has an improved biological activity, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein inosine, if present, base pairs with cytidine, and wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and X1 and/or X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, X1 and X2 each comprise at least one inosine, and X1 and X2 are each 12 nucleotides in length. In some aspects, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and X1 and X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, X1 and X2 each comprise at least one inosine, and X1 and X2 are each 12 nucleotides in length. In some aspects, N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and X1 and/or X2 each comprise at least one inosine. In some aspects, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and X1 and X2 comprise inosine and no guanosine nucleosides. In some

aspects, N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and X1 and X2 comprise inosine and no guanosine nucleosides. In some aspects, the RLR agonist of the disclosure has an improved biological activity, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonists of the disclosure comprise the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein X1 and X2 are each 12 nucleotides and comprise 1, 2, 3 or 4 inosine nucleosides. In some aspects, X1 and X2 are each 13 nucleotides and comprise 1, 2, 3, 4 or 5 inosine nucleosides. In some aspects, X1 and X2 are each 14 nucleotides and comprise 1, 2, 3, 4, 5 or 6 inosine nucleosides. In some aspects, X1 and X2 are each 15 nucleotides and comprise 1, 2, 3, 4, 5, 6, or 7 inosine nucleosides. In some aspects, X1 and X2 are each 16 nucleotides and each comprise 1, 2, 3, 4, 5, 6, 7, or 8 inosine nucleosides. In some aspects, X1 and X2 are each 12 nucleotides and comprise at least 10%, 20%, 30% or 40% inosine nucleosides. In some aspects, the RLR agonist of the disclosure has an improved biological activity, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ comprises a sequence motif $[AUCN_5]_x$ , wherein $N_5$ is comprises guanosine or inosine, wherein x is an integer whose value indicates the number of sequence motifs, and wherein x=2-4;

(viii) $X_2$ comprises a sequence motif $[CN_6AU]_y$, wherein N6 comprises guanosine or inosine, wherein y is an integer whose value indicates the number of sequence motifs, and wherein y = 2-4;

(ix) L is a linker that operably links the first polynucleotide and the second polynucleotide,

optionally, wherein at least one of N1, N2, N3, and N4 is inosine, and wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(iv) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(v) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(vi) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ comprises a sequence motif $[AUCN_5]_x$ , wherein $N_5$ is comprises guanosine or inosine, wherein x is an integer whose value indicates the number of sequence motifs, and wherein x=2-4;

(viii) $X_2$ comprises a sequence motif $[CN_6AU]_y$, wherein N6 comprises guanosine or inosine, wherein y is an integer whose value indicates the number of sequence motifs, and wherein y = 2-4;

(ix) L is a linker that operably links the first polynucleotide and the second polynucleotide,

optionally, wherein at least one of N1, N2, N3, and N4 is inosine, and wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, N5 comprises inosine and N6 comprises inosine. In some aspects, N5 comprises guanosine and N6 comprises inosine. In some aspects, N5 comprises inosine and N6 comprises guanosine. In some aspects, N5 comprises guanosine (G) and N6 comprises guanosine (G). In some aspects, x = 2 and y = 2. In some aspects, x = 3 and y = 3. In some aspects, x = 4 and y = 4. In some aspects, N1 comprises inosine (I) and N4 comprises cytidine (C). In some aspects, N2 comprises inosine (I) and N3 comprises cytidine (C). In some aspects, N3 comprises inosine (I) and N2 comprises cytidine (C). In some aspects, N4 comprises inosine (I) and N1 comprises cytidine (C). In some aspects, N1 comprises guanosine (G). In some aspects, N2 comprises guanosine (G). In some aspects, N1 comprises cytidine (C). In some aspects, N2 comprises cytidine (C). In some aspects, N1 and N2 comprise guanosine (G) and N3 and N4 comprise cytidine (C). In some aspects, N1 and N2 comprise cytidine (C) and N3 and N4 comprise guanosine (G). In some aspects, N1 and N2 comprise inosine (I) and N3 and N4 comprise cytidine (C). In some aspects, N1 and N2 comprise cytidine (C) and N3 and N4 comprise inosine (I). In some aspects, the RLR agonist of the disclosure has an improved biological activity, wherein the improved biological activity is an increase in RLR-mediated cytokine production; an increase in RLR-mediated expression of interferon-stimulated genes; an increase in RLR-mediated

intracellular signaling; an increase in binding affinity to RLRs; and a combination of any of the foregoing.

In any of the foregoing or related aspects, the RLR agonist of the disclosure comprises a linker, wherein the linker is a nucleotide linker or a non-nucleotide linker. In some aspects, the linker is a non-nucleotide linker. In some aspects, the linker is a nucleotide linker. In some aspects, the nucleotide linker comprises a tetraloop, wherein the nucleotide sequence of the tetraloop is selected from the group consisting of:

(a) UNCG, wherein N = A, C, G, or U;

(b) GNRA, wherein N = A, C, G, or U, and wherein R = A or G;

(c) ANYA, wherein N = A, C, G, or U, and wherein Y = C or T;

(d) CUYG, wherein Y = C or T;

(e) UMAC, wherein M = A or C; and

(f) CUUG.

In some aspects, the sequence of the tetraloop is UUCG. In some aspects, the sequence of the tetraloop is GAUC.

In any of the foregoing or related aspects, the RLR agonist of the disclosure comprises a nucleotide linker, wherein the nucleotide linker comprises the nucleotide sequence UUUGAU or UGUUU. In some aspects, the nucleotide linker comprises the nucleotide sequence UUUGAU. In some aspects, the nucleotide linker comprises the nucleotide sequence UGUUU.

In any of the foregoing or related aspects, the RLR agonist of the disclosure comprises a non-nucleotide linker, wherein the non-nucleotide linker is selected from the group consisting of:

(a) an ethylene glycol linker; and

(b) an alkyl linker.

In some aspects, the non-nucleotide linker is a hexaethylene glycol linker. In some aspects, the non-nucleotide linker is a C9 alkyl linker.

In any of the foregoing or related aspects, the RLR agonist of the disclosure comprises a 5’ diphosphate moiety, or a derivative or analog thereof. In some aspects, the agonist comprises a 5’ triphosphate moiety, or a derivative or analog thereof. In some aspects, the derivative or analog thereof comprises a phosphate bioisostere is selected from: a phosphonate, a thiophosphonate, a phosphorothioate, a sulfate, a sulfonate, a sulfamate, a thiazolidinone, a carboxylate, a malonate, a boronic acid, a benzoxaborole, a boranophosphate, a squaramide.

In any of the foregoing or related aspects, the RLR agonist of the disclosure comprises a modified nucleotide, a modified nucleoside, or a modified nucleobase, or a combination thereof. In some aspects, the agonist comprises a modification to the internucleotide linkages or to the polynucleotide backbone.

In any of the foregoing or related aspects, the RLR agonist of the disclosure exhibits one or more of the following properties:

(a) specifically binds to one or more RLRs (e.g. RIG-1, MDA5 and/or LGP2);

(b) increases RLR-mediated cytokine production;

(c) increases RLR-mediated expression of interferon-stimulated genes (ISGs);

(d) increases RLR-dependent intracellular signaling;

(e) increases stability of the duplex;

(f) increases binding affinity to RLRs;

(g) decreases off-target binding;

(h) increases biological half-life;

(i) increases biodistribution and bioavailability;

(j) increases and/or enhances uptake into cells and/or tissues;

(k) decreases immunogenicity; and

(l) a combination of any of (a)-(k).

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 each comprise guanosine, wherein N3 and N4 each comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, wherein X1 and X2 each comprise at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and wherein L comprises a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is UUCG,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 each comprise guanosine, wherein N3 and N4 each comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, wherein X1 and X2 each comprise at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and wherein L comprises a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is UUCG,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein N1 comprises inosine and N2 comprise guanosine, wherein N3 and N4 each comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, wherein X1 and X2 each comprise at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and wherein L comprises a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is UUCG,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein N1 comprises inosine and N2 comprise guanosine, wherein N3 and N4 each comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, wherein X1 and X2 each comprise at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and wherein L comprises a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is UUCG,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, wherein X1 and X2 each comprise at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and wherein L comprises a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is UUCG,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, wherein X1 and X2 each comprise at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and wherein L comprises a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is UUCG,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 comprise guanosine, wherein N3 and N4 comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, and wherein the non-nucleotide linker is a C9 alkyl linker,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 comprise guanosine, wherein N3 and N4 comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, and wherein the non-nucleotide linker is a hexaethylene glycol linker,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLR, wherein the 5' most nucleotide of the agonist comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 and 36,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 22, 23 and 25,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 58 and 89, respectively;

(ii) SEQ ID NO: 59 and 89, respectively; and

(iii) SEQ ID NO: 61 and 91, respectively.

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively,

wherein the at least one RLR agonist is packaged in the virus like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 comprise guanosine, wherein N3 and N4 comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, and wherein the non-nucleotide linker is a C9 alkyl linker,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein N1 and N2 comprise guanosine, wherein N3 and N4 comprise cytidine, wherein X1 and X2 are each 12 nucleotides in length, and wherein the non-nucleotide linker is a hexaethylene glycol linker,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLR, wherein the 5' most nucleotide of the agonist comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 and 36,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence

motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 22, 23 and 25,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 58 and 89, respectively;

(ii) SEQ ID NO: 59 and 89, respectively; and

(iii) SEQ ID NO: 61 and 91, respectively.

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first

polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively,

wherein the at least one RLR agonist is packaged in the virus like particle.

In any of the foregoing or related aspects, the nucleotide sequence comprising the RLR agonist is not complementary to a genomic DNA sequence or mRNA sequence, wherein the RLR agonist does not participate in RNA interference, and wherein the RLR agonist does not silence gene expression.

In any of the foregoing or related aspects, a virus-like particle described herein lacks a lipoprotein-containing envelope.

In any of the foregoing or related aspects, a virus-like particle described herein is a recombinant virus-like particle. In some aspects, the recombinant virus-like particle is selected from the group consisting of:

(a) recombinant proteins of Hepatitis B virus;

(b) recombinant proteins of measles virus;

(c) recombinant proteins of Sinbis virus;

(d) recombinant proteins of Rotavirus;

(e) recombinant proteins of Foot-and-Mouth-Disease virus;

(f) recombinant proteins of Retrovirus;

(g) recombinant proteins of Norwalk virus;

(h) recombinant proteins of human Papilloma virus;

(i) recombinant proteins of BK virus;

(j) recombinant proteins of bacteriophages;

(k) recombinant proteins of RNA-phages;

(1) recombinant proteins of Qβ-phage;

(m) recombinant proteins of GA-phage

(n) recombinant proteins of fr-phage;

(o) recombinant proteins of AP 205-phage;

(p) recombinant proteins of Ty; and

(q) fragments of any of the recombinant proteins from (a) to (p).

In any of the foregoing or related aspects, a virus-like particle described herein comprises recombinant proteins of an RNA-phage, wherein said RNA-phage is selected from the group consisting of: (a) bacteriophage Qβ; (b) bacteriophage R17; (c) bacteriophage fr; (d) bacteriophage GA; (e) bacteriophage SP; (f) bacteriophage MS2; (g) bacteriophage M11; (h) bacteriophage MX1; (i) bacteriophage NL95; (j) bacteriophage f2; (k) bacteriophage PP7; and (l) bacteriophage AP205.

In any of the foregoing or related aspects, a virus-like particle described herein comprises recombinant proteins of bacteriophage Qβ. In some aspects, the recombinant proteins of bacteriophage Qβ comprise coat proteins having the amino acid sequence of SEQ ID NO: 112. In some aspects, the recombinant proteins of bacteriophage Qβ comprise coat proteins having an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 112.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ comprising coat proteins having the amino acid sequence of SEQ ID NO: 112; and

(b) at least one synthetic RIG-I like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the RLR agonist comprises a ribonucleic acid (RNA) of 10-100 nucleotides in length, wherein the 5' most nucleotide of the RNA comprises a 5'diphosphate or triphosphate moiety, or derivative or analog thereof,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In some aspects, the disclosure provides a composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RIG-I like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist the nucleotide sequence of SEQ ID NO: 23, and wherein the 5' most nucleotide of the agonist comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof,

wherein the at least one RLR agonist is packaged in the virus-like particle.

In any of the foregoing or related aspects, the RLR agonist is non-covalently bounds to the virus-particle.

In any of the foregoing or related aspects, the RLR agonist is bound to a virus-like particle site selected from the group consisting of an oligonucleotide binding site, a DNA binding site and an RNA binding site. In some aspects, the virus-like particle comprises an arginine-rich repeat.

In any of the foregoing or related aspects, the compositions described herein comprise at least one antigen or antigenic determinant bound to a virus-like particle. In some aspects, the at least one antigen or antigenic determinant is bound to the virus-like particle by at least one covalent bond. In some aspects, the at least one antigen or antigenic determinant is bound to the virus-like particle by a non-peptide bond. In other aspects, the antigen or antigenic determinant is fused to the virus-like particle. In some aspects, the virus-like particle comprises at least one first attachment site and wherein the antigen or antigenic determinant comprises at least one second attachment site selected from the group consisting of (a) an attachment site not naturally occurring within the antigen or antigenic determinant; and (b) an attachment site naturally occurring within the antigen or antigenic determinant, and

wherein the binding of the antigen or antigenic determinant to the virus-like particle is effected through association between the first attachment site and the second attachment site, optionally wherein the association is through at least one non-peptide bond. In some aspects, the first attachment site comprises an amino group or a lysine residue, and wherein the second attachment site comprises a sulfhydryl group or a cysteine residue.

In some aspects, the disclosure provides a pharmaceutical composition for stimulating an immune response, treating or delaying progression of a cancer, or reducing or inhibiting tumor growth in a subject in need thereof, comprising a composition provided by the disclosure, and a pharmaceutically acceptable carrier. In some aspects, the composition is formulated in a polyethylenimine (PEI) carrier. In some aspects, the PEI carrier is JetPEI®.

In some aspects, the disclosure provides a method to increase RLR-mediated production of one or more cytokines in a cell, the method comprising contacting the cell with a composition provided by the disclosure, wherein the composition increases RLR-mediated cytokine production in a cell. In some aspects, the composition increases RLR-mediated type I interferon (e.g., IFN-α, IFN-β) production in a cell. In some aspects, the composition increases RLR-mediated IL-1β production in a cell. In some aspects, the composition increases RLR-mediated IP-10 production in a cell. In some aspects, the composition increases RLR-mediated IL-6, IL-12p70, MCP-1 and/or MIP-1β production in a cell.

In some aspects, the disclosure provides a method to increase RLR-mediated expression of one or more interferon-stimulated genes in a cell, the method comprising contacting the cell with a composition provided by the disclosure, wherein the composition increases RLR-mediated expression of one or more interferon-stimulated genes in a cell.

In some aspects, the disclosure provides a method to increase RLR-dependent intracellular signaling in a cell, the method comprising contacting the cell with a composition provided by the disclosure, wherein the composition increases RLR-dependent intracellular signaling.

In some aspects, the disclosure provides a method of stimulating an immune response in a subject, the method comprising administering to the subject an effective amount of a composition provided by the disclosure.

In some aspects, the disclosure provides a method of treating or delaying progression of a cancer in a subject, the method comprising administering to the subject an effective amount of a composition provided by the disclosure.

In some aspects, the disclosure provides a method of reducing or inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition provided by the disclosure.

In some aspects, the disclosure provides a method for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject an effective amount of a composition provided by the disclosure, wherein the composition, increases RLR-mediated production of one or more cytokines in a cell, increases RLR-mediated expression of one or more interferon-stimulated genes in a cell, and or increases RLR-dependent intracellular signaling in a cell, thereby stimulating the immune response, treating or delaying progression of the cancer, or inhibiting growth of the tumor.

In some aspects of, a composition provided by the disclosure is administered in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a cytokine, an activator or agonist of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof.

In some aspects, a composition provided by the disclosure is administered preceding or subsequent to administration of the one or more additional therapeutic agents or wherein the one or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the agonist or pharmaceutical composition.

In some aspects, the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist, a TIM-3 antagonist, a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, a KIR antagonist, a LAG-3 antagonist, a Toll-like receptor 3 (TLR3) agonist, a Toll-like receptor 7 (TLR7) agonist, a Toll-like receptor 9 (TLR9) agonist.

In some aspects, the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD137 (4-1BB).

In some aspects, the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD134 (OX40).

In some aspects, the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist. In some aspects, the PD-1/PD-L1 antagonist is selected from the group consisting of: PDR001, KEYTRUDA® (pembrolizumab), OPDIVO® (nivolumab), pidilizumab, MEDI0680, REGN2810, TSR-042, PF-06801591, and AMP-224. In some aspects, the PD-1/PD-L1 antagonist is selected from the group consisting of: FAZ053, TENCENTRIQ® (atezolizumab), BAVENCIO® (avelumab), IMFINZI® (durvalumab), and BMS-936559.

In some aspects, the one or more additional therapeutic agents is a TIM-3 antagonist.

In some aspects, the one or more additional therapeutic agents is a VISTA antagonist.

In some aspects, the one or more additional therapeutic agents is an adenosine A2AR antagonist.

In some aspects, the one or more additional therapeutic agents is a B7-H3 antagonist.

In some aspects, the one or more additional therapeutic agents is a B7-H4 antagonist.

In some aspects, the one or more additional therapeutic agents is a BTLA antagonist.

In some aspects, the one or more additional therapeutic agents is a CTLA-4 antagonist.

In some aspects, the one or more additional therapeutic agents is a IDO antagonist.

In some aspects, the one or more additional therapeutic agents is a KIR antagonist.

In some aspects, the one or more additional therapeutic agents is a LAG-3 antagonist.

In some aspects, the one or more additional therapeutic agents is a Toll-like receptor 3 (TLR3) agonist. In some aspects, the TLR3 agonist is polyinosinic:polycytidylic acid (poly I:C). In some aspects, the TLR3 agonist is HILTONOL® (poly ICLC). In some aspects, the TLR3 agonist is polyadenylic-polyuridylic acid (poly A:U). In some aspects, the TLR3 agonist is RIBOXXIM® (RGIC®100). In some aspects, the TLR3 agonist is RIBOXXON® (RGIC®50 bioconjugate). In some aspects, the TLR3 agonist is RIBOXXOL® (RGIC®50).

In some aspects, the one or more additional therapeutic agents is a Toll-like receptor 7 (TLR7) agonist. In some aspects, the TLR7 agonist is GS-9620 (Vesatolimod). In some aspects, the TLR7 agonist is imiquimod (ALDARA$^{TM}$). In some aspects, the TLR7 agonist is resiquimod (R-848).

In some aspects, the one or more additional therapeutic agents is a Toll-like receptor 9 (TLR9) agonist. In some aspects, the TLR9 agonist is a CpG oligodeoxynucleotide (CpG ODN). In some aspects, the CpG ODN is a Class A CpG ODN (CpG-A ODN). In some aspects, the CpG ODN is a Class B CpG ODN (CpG-B ODN). In some aspects, the CpG ODN is a Class C CpG ODN (CpG-C ODN).

In some aspects, the disclosure provides a use a composition provided by the disclosure, for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, optionally for use in combination with one or more additional therapeutic agents.

In some aspects, the disclosure provides a use of a composition provided by the disclosure, in the manufacture of a medicament for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, optionally for use in combination with one or more additional therapeutic agents. In some aspects, the composition is administered in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a cytokine, an activator of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof. In some aspects, the composition is administered preceding or subsequent to administration of the one or more additional therapeutic agents or

wherein the one or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the composition.

In some aspects, the disclosure provides a kit comprising a composition provided by the disclosure and instructions for use in stimulating an immune response in a subject, or treating or delaying progression of a cancer, or inhibiting tumor growth in a subject, optionally with instructions for use in combination with one or more additional therapeutic agents. In some aspects, the kit comprises instructions for administering the composition in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a cytokine, an activator of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof. In some aspects, the composition is administered preceding or subsequent to administration of the one or more additional therapeutic agents or wherein the one or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the composition.

In any of the foregoing or related aspects, the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist, a TIM-3 antagonist, a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, a KIR antagonist, a LAG-3 antagonist, a Toll-like receptor 3 (TLR3) agonist, a Toll-like receptor 7 (TLR7) agonist, a Toll-like receptor 9 (TLR9) agonist.

In any of the foregoing or related aspects, the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD137 (4-1BB).

In any of the foregoing or related aspects, the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD134 (OX40).

In some aspects, the disclosure provides a method of producing a composition as described herein, the method comprising:

(a) disassembling the virus-like particle;

(b) adding the RLR agonist; and

(c) reassembling the virus-like particle.

In some aspects, the method comprises removing nucleic acids of the disassembled virus-like particle. In some aspects, the method comprises purifying the composition after reassembly. In some aspects, the method comprises (d) binding an antigen or antigenic determinant to the virus-like particle. In some aspects, the antigen or antigenic determinant is bound to the virus-like particle before disassembling the virus-like particle. In other aspects, the antigen or antigenic determinant is bound to the virus-like particle after reassembling the virus-like particle.

## BRIEF DESCRIPTION OF THE DRAWINGS

**FIG. 1** provides a bar graph depicting the quantification of cytokine secretion: IFN-α2a from human PBMCs treated with 0.4 nM, 2 nM, and 10 nM of RLR agonists comprising various modifications.

**FIG. 2** provides a bar graph depicting the quantification of IFN-α secretion from human PBMCs treated with RIG 50c (X24907) and the inosine-substituted RIG 27c (X24935) at concentrations of 0.2 nM, 2 nM, 20 nM, and 200 nM.

**FIG. 3** provides a bar graph depicting the quantification of IFN-α secretion from human PBMCs treated with Qβ-RIG27 (RIG 27c packaged into a VLP with RNA-phage Qβ coat protein) at concentrations of 2 nM, 20 nM, 200 nM, and 600 nM.

## DETAILED DESCRIPTION

### Overview

The RIG-I-like receptors (RLRs) are a family of cytosolic pattern recognition receptors that are essential for detecting viral RNA and initiating the innate immune response. The RLR family includes three members: Retinoic acid-inducible gene I (RIG-I), Melanoma differentiation-associated gene 5 (MDA5), and Laboratory of genetics and physiology 2 (LGP2). These receptors are expressed in both immune and non-immune cell types and regulate signaling pathways that promote the IRF3-, IRF7-dependent expression of type I and type III interferons (IFNs), and the NF-kappa B-dependent expression of pro-inflammatory cytokines.

All three RLR family receptors have a DExD/H box RNA helicase domain with ATPase activity. This domain along with the adjacent C-terminal domain is required for RNA binding. In addition, the C-terminal domains of RIG-I and LGP2 have been shown to act as repressor domains,

ensuring that the receptors remain in an inactive conformation until they are bound by an activating RNA.

The present disclosure provides RLR agonists comprising synthetic RNA molecules that fold to form a duplexed, dsRNA and that comprise one or more sequence motifs that provides one or more improved biological activities. The present disclosure also provides a composition comprising at least one RLR agonist packaged into VLPs, and demonstrates improved immunostimulatory potency, e.g. induction of cytokine expression, compared to VLPs alone. The RIG-VLPs provides an improved immunostimulatory compositions for use in prophylactic or therapeutic regimens against, e.g., tumors.

**RIG-I-like Receptors and Their Ligands**

The present disclosure provides synthetic RNA ligands that specifically bind to RIG-I-like receptors (RLRs) and agonize RLRs (RLR agonists). In some aspects, the disclosure provides RLR agonists that are useful for the treatment of cancer. In some aspects, the disclosure provides RLR agonists that are useful for the treatment of infectious disease. In some embodiments, the RLR agonists induce cytokine production. In some embodiments, the RLR agonists increase the number of CD8+ T cells in the tumor microenvironment. In some embodiments, the RLR agonists induce protective anti-tumor immunity

RIG-I-like receptors (RLRs) comprise a family of DExD/H box RNA helicases that function as cytosolic pattern recognition receptors (PRRs) that sense the presence of pathogenic agents via the recognition of pathogen-associated molecular patterns (PAMPs). In particular, the intracellular presence of non-self (e.g., viral) RNA is sensed by an infected cell via binding of the RNA to RLRs and results in the initiation and modulation of antiviral immunity. Like most viral RNAs, endogenous mRNA and RNA polymerase III transcripts are also 5'-triphosphorylated, but eukaryotic mRNAs possess a 5' cap structure linked to a guanosine methylated at N7 that prevents RIG-I activation. These structural differences between viral and self RNAs, together with differences in intracellular localization, are thought to enable the effective function of RIG-I as a defense against viral infection by the preferential detection of viral RNA. The molecular recognition and binding of non-self RNA ligands to RLRs propagates specific intracellular signal events culminating in the activation of transcription factors that drive type 1 interferon (IFN) production and antiviral gene expression. The RLR-mediated induction of IFN and inflammatory

cytokines production as well as antiviral gene expression elicits an immune response to control virus infection (Yoneyama et al., (2015) Curr Opin Immunol 32:48-53).

Three RLR family members have been identified: RIG-I (retinoic acid-inducible gene I) – the founding member and best characterized of RLR family, MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2). RIG-I is an important component of the innate immune system and plays a critical role in the defense against infection by RNA viruses. In contrast to the Toll-like receptors TLR3, TLR7, TLR8, and TLR9, that detect nucleic acids in the endosomes of a subset of immune cells, RIG-I is a cytosolic innate immune receptor that is expressed in all cell types (Kato et al., (2006) Nature 441(7089):101-105; Loo et al., (2008) J Virol 82(1):335-345). Two early studies independently established that RIG-I specifically detects and is activated by viral RNAs (Hornung et al., (2006) Science 314(5801):994-997; Pichlmair et al., (2006) Science 314(5801):997-1001).

High-resolution structures of RIG-I/ligand complexes have provided the molecular detail of RIG-I binding to RNA ligands, specifically to the activating ligand, double-stranded 5'-triphosphorylated RNA (ppp-dsRNA) (Civril et al., (2011) EMBO Reports 12(11):1127-1134; Jiang et al., (2011) Nature 479(7373):423-427; Kowalinski et al., (2011) Cell 147(2):423-435; Lu et al., (2010) Structure 18(8): 1032-1043; Luo et al., (2011) Cell 147(2) 409-422; Wang et al., (2010) Nature Structural & Molecular Biology 17(7):781-787; Hornung et al., (2006) Science 314(5801):994-997; Pichlmair et al., (2006) Science 314(5801):997-1001; Schlee et al., (2009) Immunity 31(1):25-34). ). The crystal structures of RIG-I/RNA complexes show protein binding to the backbone, not the bases, suggesting that the RNA sequence may not affect RIG-I binding or that RNA sequence may exhibit as of yet uncharacterized effects or activity. To date, evidence for sequence–dependent differential interaction or affinity with, and activation of, RIG-I-like receptors is not described in the art (Schlee and Hartmann (2010) Molecular Therapy 18(7):1254-1262).

Accordingly, the disclosure provides synthetic RIG-I-like receptor (RLR) agonists comprising non-naturally occurring, synthetic, and or engineered RLR RNA ligands. In some embodiments, the RLR agonist comprises a ribonucleic acid (RNA) of 10-100 nucleotides in length. In some aspects the RNA is 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, or 95-100 nucleotides in length.

In some embodiments, the RLR agonist may be single-stranded, single-stranded containing a self-complementary sequence which can form a duplex, a stem-loop or a hairpin structure, double-stranded, or partially double-stranded oligonucleotide.

In some embodiments, the double-strand oligonucleotide is fully double-stranded. In this case, the oligonucleotide is composed of two single-stranded oligonucleotides which have the same length and which have sequences that are 100% complementary to each other.

In some embodiments, the double-strand oligonucleotide is partially double-stranded. In this case, the two strands forming the oligonucleotide have different lengths, sequences which are not 100% complementary to each other, or both. In other words, the at least one fully double-stranded section of the oligonucleotide is connected with a single-stranded structure at one or both ends.

In some embodiments the duplex, hairpin, or stem-loop structure comprises 10-15, 15-20, 20-25, 25-30, 30-35, 30-35, 35-40, 40-45, 45-50, 50-55 base pairs.

In some embodiments, the oligonucleotide forms a duplex comprising less than 19 base pairs. In some embodiments, the complementary bases of the duplex are connected by a nucleotide or non-nucleotide linker

In some embodiments, the oligonucleotide is single-stranded, single-stranded containing a self-complementary sequence or double-stranded, the length of the oligonucleotide is the length of a single-strand.

In some aspects, the oligonucleotide is partially double-stranded, the length of the oligonucleotide is the length of the longer strand. Therefore, the oligonucleotide of the present invention includes partially double-stranded oligonucleotides wherein at least one of the strands is at 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, or 85-90 nucleotides in length.

In some aspects, the oligonucleotide is double-stranded or partially double-stranded oligonucleotide, at least one of the strands comprises at least one 5' di- or tri-phosphate group. When both strands comprise 5' di- or tri-phosphate groups, the number of phosphate groups may be the same or may be different on the two strands. In some aspects, the oligonucleotide is a partially double-stranded oligonucleotide, the at least 1 ribonucleotide at the 5' end which comprises the at least one 5' diphosphate or triphosphate can be on either the long or the short

strand, wherein at least the long strand is 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, or 85-90 nucleotides in length.

In some aspects, the degree of complementarity is preferably at least 50%, 60%, 70%, more preferably at least 75%, 80%, 85%, 90%, even more preferably at least 95%, 96%, 97%, 98%, 99%, and most preferably 100%. As used in the art, the term "degree of complementarity" between two oligonucleotides/polynucleotides refers to the percentage of complementary bases in the overlapping region of the two oligonucleotides. Two bases are complementary to each other if they can form a base pair via hydrogen bonding. Base pairs include both Watson-Crick base pairs and wobble base pairs. Watson-Crick base pairs include A-T, C-G, A-U; wobble base pairs include G-U, I-U, I-A, I-C. The degree of complementarily can be determined by a skilled person using any known methods in the art, either manually or automatically by various engines such as BLAST. For example, ATCG has 100% complementarity to CGAT and CGATGG, and 75% complementarity to CGTT and CGTTGG.

In some aspects, the disclosure provides an RLR agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first polynucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif.

In some embodiments, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is selected from the group consisting of:

(i) a GT-repeat motif;

(ii) a GA-repeat motif;

(iii) a AUCG-repeat motif;

(iv) an AU-repeat motif;

(v) a dipyrimidine motif;

(vi) a dipurine motif;

(vii) a pyrimidine triplet motif;

(viii) a purine triplet motif;

(ix) a palindromic sequence motif; and

(x) a combination of any of (i)-(ix).

In some embodiments, the RLR agonists of the disclosure comprise at least one improved biological activity, wherein the improved biological activity is selected from:

(i) an increase in RLR-mediated cytokine production;

(ii) an increase in RLR-mediated expression of interferon-stimulated genes;

(iii) an increase in RLR-mediated intracellular signaling;

(iv) an increase in binding affinity to RLRs; and

(v) a combination of any of (i)-(iv).

In some embodiments, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a GT-repeat motif comprises a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 5-10, about 5, about 4 about 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and thymine nucleotides, or derivatives or analogs thereof. In some embodiments, the GT-repeat motif is $[GT]_n$, wherein n = 2 to 9. In some embodiments, the GT-repeat motif is $[GT]_7$. In some embodiments, the GT-repeat motif is $[GT]_3$, and wherein the GT-repeat motif is followed by a purine triplet and UCG, respectively. In some embodiments, the purine triplet is GGA.

In some embodiments, the sequence motif is a GA-repeat motif comprises a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 5-10, about 5, about 4 about 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and adenine nucleotides, or derivatives or analogs thereof. In some embodiments, the GA-repeat motif is $[GA]_n$, where n = 2 to 9. In some embodiments, the GA-repeat motif is $[GA]_7$.

In some embodiments, the RLR agonists of the disclosure comprise a sequence motif, wherein the sequence motif is a AUCG-repeat motif comprising a sequence of <19, about 16, about 12-16, about 12, about 8-12, about 6, about 16, 12, 8 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof.

In some embodiments, the AUCG-repeat motif is $[AUCG]_n$, where n = 2 to 4. In some embodiments, the AUCG-repeat motif is $[AUCG]_3$.

In some embodiments, the AUCG-repeat motif is preceded by a CG or a dipyrimidine motif. In some embodiments, the AUCG-repeat motif is preceded by a CG. In some embodiments, the dipyrimidine motif is CC. In some embodiments, the AUCG-repeat motif is preceded by a

dipurine motif. In some embodiments, the dipurine motif is GA. In some embodiments, the dipurine motif is GG.

In some embodiments, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein one or more uridine nucleosides (U) are substituted with a modified nucleoside. In some embodiments, wherein the modified nucleoside is ribothymidine (T). In some embodiments, the AUGC-repeat motif is $[AUCG]_3$, wherein the one or more uridine nucleosides (U) comprising the AUCG-repeat motif are substituted with a modified nucleoside, wherein the modified nucleoside is ribothymidine (T). In some embodiments, the AUGC-repeat motif is $[AUCG]_3$, wherein the one or more uridine nucleosides (U) comprising the AUCG-repeat motif are substituted with a modified nucleoside, wherein the modified nucleoside is ribothymidine (T), and wherein the AUGC-repeat motif is preceded by GG.

In some embodiments, the RLR agonists of the disclosure comprise an AUCG-repeat motif, wherein one or more guanosine nucleosides (G) are substituted with a modified nucleoside. In some embodiments, the modified nucleoside is inosine (I). In some embodiments, the AUGC-repeat motif is $[AUCG]_3$, wherein the one or more guanosine nucleosides (G) comprising the AUCG-repeat motif are substituted with a modified nucleoside, wherein the modified nucleoside is ribothymidine (T), and wherein the AUGC-repeat motif is preceded by GG.

In some embodiments, the RLR agonists of the disclosure comprise a AUCG-repeat motif, wherein the motif is preceded by a IG. In some embodiments, the AUCG-repeat motif is $[AUCG]_3$ and is preceded by a IG.

In some embodiments, the RLR agonists of the disclosure comprise an AUCG-repeat, wherein one or more guanosine nucleosides (G) are substituted with an inosine (I), wherein the AUCG-repeat is preceded by an inosine (I). In some embodiments, the guanosine nucleosides (G) comprising the AUCG-repeat are substituted with an inosine (I), wherein the AUCG-repeat is preceded by an inosine (I), wherein the 5' most nucleotide of the first polynucleotide comprises inosine (I).

In some embodiments, the 5' most nucleotide of the first polynucleotide comprises inosine (I).

In some embodiments, the RLR agonists of the disclosure comprise a AUCG-repeat sequence motif, wherein the AUCG-repeat motif is $[AUCG]_2$. In some embodiments, the AUCG-repeat motif is preceded by a dipurine motif. In some embodiments, the dipurine motif is GG. In

some embodiments, the AUCG-repeat motif is preceded by a purine triplet. In some embodiments, the purine triplet is GGG. In some embodiments, the AUCG-repeat motif is preceded by CCCCCG. In some embodiments, the AUCG-repeat motif is preceded by TCGUCG.

In some embodiments, the RLR agonists of the disclosure comprise a palindromic sequence, wherein the palindromic sequence comprises a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome.

In some embodiments, the linker is flanked by AU. In some embodiments, the linker is flanked by an AU-repeat motif, wherein the AU-repeat motif is $[AU]_n$, where n = 2 to 3. In some embodiments, the AU-repeat motif is $[AU]_2$.

In some aspects, the disclosure provides an RLR agonist that specifically binds to a RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that covalently links the first polynucleotide and the second polynucleotide.

In other aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to RIG-I-like receptors (RLRs), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide.

In some embodiments, inosine, if present in the RLR agonist, base pairs with cytidine.

In some embodiments, the linker (L) is a nucleotide linker or a non-nucleotide linker.

In some aspects, the disclosure provides an RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a nucleotide or non-nucleotide linker. RNA hairpins are among the most common RNA secondary structural elements, wherein the hybridized portion or "stem" of the hairpin are frequently capped by RNA tetraloops.

RNA tetraloops are composed of characteristic four-loop nucleotides that form a compact and stable structure. While they can be formed by many different nucleotide sequences, UNCG (N = A, C, G, or U), GNRA (R = A or G), and CUUG tetraloops are found most often. Tetraloops usually help initiate RNA-folding processes and provide sites for tertiary contacts within or between RNAs and for protein binding, thereby facilitating the assembly of ribonucleoprotein particles. Further description of tetraloops can be found in Cheong, H. , Kim, N. and Cheong, C. (2015). RNA Structure: Tetraloops. In eLS, John Wiley & Sons, Ltd (Ed.), which is incorporated herein by reference in its entirety.

Accordingly, in some embodiments, the RLR agonists of the disclosure comprise a nucleotide linker comprising a tetraloop. In some embodiments, the nucleotide sequence of the tetraloop is selected from the group consisting of:

(a) UNCG, wherein N = A, C, G, or U;

(b) GNRA, wherein N = A, C, G, or U, and wherein R = A or G;

(c) ANYA, wherein N = A, C, G, or U, and wherein Y = C or T;

(d) CUYG, wherein Y = C or T;

(e) UMAC, wherein M = A or C; and

(f) CUUG.

In some embodiments, the nucleotide linker comprises the nucleotide sequence UUUGAU or UGUUU. In some embodiments, the sequence of the tetraloop is UUCG. In some embodiments, the sequence of the tetraloop is GAUC. In some embodiments, the nucleotide linker comprises the nucleotide sequence UUUGAU. In some embodiments, the nucleotide linker comprises the nucleotide sequence UGUUU.

In other aspects, the RLR agonists of the disclosure comprise a non-nucleotide linker. As described herein nucleic acid loops (e.g., tetraloops) are a common element found in nucleic acid secondary structure. Nucleotide loops arise in folded domains occurring in intrastrand duplexes. Synthetic nucleic acids designed to contain hairpin loops comprising non-nucleotide linking groups (e.g., non-nucleotide linkers) can replace several nucleotides bridging a folded duplex structure. Non-nucleotide groups have been used as linkers in non-folded structures as well. Such linking groups may be useful replacements of natural nucleotide linkers (e.g., tetraloops). For example, they can shorten the synthesis of nucleic acid with a desired secondary structure by several steps, since one relatively long non-nucleotide linking group replaces several individual

nucleotides which may normally constitute a loop. Such non-natural loops or linkers (e.g., non-nucleotide linkers) can confer resistance to degradation by nucleases which would ordinarily act on a natural loop structure in biological contexts (e.g., in a cell or in the circulation of a subject upon administration). A non-nucleotide linking group also has the potential to provide a more stable folded structure than occurs with the nucleotide loops and or linkers. Further description of non-nucleotide linkers can be found in Rumney and Kool (1995) J Am Chem Soc 117:5635-5646, which is incorporated herein by reference in its entirety.

Accordingly, in some embodiments, the RLR agonists of the disclosure comprise a non-nucleotide linker selected from the group consisting of:

(a) an ethylene glycol linker; and

(b) an alkyl linker.

In some embodiments, the non-nucleotide linker is a hexaethylene glycol linker. In some embodiments, the non-nucleotide linker is a C9 alkyl linker.

In some embodiments, the RLR agonist comprises a 5’ diphosphate moiety, or a derivative or analog thereof. In some embodiments, the agonist comprises a 5’ triphosphate moiety, or a derivative or analog thereof. In some embodiments, the derivative or analog thereof comprises a phosphate bioisostere is selected from: a phosphonate, a thiophosphonate, a phosphorothioate, a sulfate, a sulfonate, a sulfamate, a thiazolidinone, a carboxylate, a malonate, a boronic acid, a benzoxaborole, a boranophosphate, a squaramide.

In some embodiments, the agonist comprises a modified nucleotide, a modified nucleoside, or a modified nucleobase, or a combination thereof. In some embodiments, the agonist comprises a modification to the internucleotide linkages or to the polynucleotide backbone.

In some aspects, the RLR agonist of the disclosure exhibits at least one or more of the following properties:

(a) specifically binds to one or more RLRs (e.g. RIG-1, MDA5 and/or LGP2);

(b) increases RLR-mediated cytokine production;

(c) increases RLR-mediated expression of interferon-stimulated genes (ISGs);

(d) increases RLR-dependent intracellular signaling;

(e) increases stability of the duplex;

(f) increases binding affinity to RLRs;

(g) decreases off-target binding;

(h) increases biological half-life;

(i) increases biodistribution and bioavailability;

(j) increases and/or enhances uptake into cells and/or tissues;

(k) decreases immunogenicity; and

(l) a combination of any of (a)-(k).

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein at least one of N1, N2, N3, and N4 is inosine and/or at least one of X1 and/or X2 comprises at least one inosine nucleoside, and wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA.

In some embodiments, N1 comprises inosine and N4 comprises cytidine. In some embodiments, N1 comprises cytidine and N4 comprises inosine. In some embodiments, N2 comprise inosine and N3 comprises cytidine. In some embodiments, N2 comprises cytidine and N3 comprises inosine. In some embodiments, N1 comprises guanosine. In some embodiments, N2 comprises guanosine. In some embodiments, N1 comprises cytidine. In some embodiments, N2 comprises cytidine. In some embodiments, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine. In some embodiments, N1 and N2 comprise cytidine and N3 and N4 comprise guanosine. In some embodiments, N1 and N2 comprise inosine and N3 and N4 comprise cytidine. In some embodiments, N1 and N2 comprise cytidine and N3 and N4 comprise inosine. In some embodiments, N1 comprises inosine and N4 comprises cytidine, and X1 and/or X2 each comprise at least one inosine. In some embodiments, N2 comprises inosine and N3 comprises cytidine, and X1 and/or X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise guanosine N3 and N4 comprise cytidine, and X1 and/or X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and X1 and X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and X1 and X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and X1 and X2 each comprise inosine and no guanosine nucleosides. In some embodiments, N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and X1 and X2 each comprise inosine and no guanosine nucleosides. In some embodiments, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and X1 and/or X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and X1 and X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and X1 and/or X2 each comprise at least one inosine. In some embodiments, N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and X1 and X2 comprise inosine and no guanosine nucleosides. In some embodiments, N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and X1 and X2 comprise inosine and no guanosine nucleosides. In some embodiments, X1 and X2 are each 12 nucleotides and comprise 1, 2, 3 or 4 inosine nucleosides. In some embodiments, X1 and X2 are each 13 nucleotides and comprise 1, 2, 3, 4 or 5 inosine nucleosides. In some embodiments, X1 and X2 are each 14 nucleotides and comprise 1, 2, 3, 4, 5 or 6 inosine nucleosides. In some embodiments, X1 and X2 are each 15 nucleotides and comprise

1, 2, 3, 4, 5, 6, or 7 inosine nucleosides. In some embodiments, X1 and X2 are each 16 nucleotides and each comprise 1, 2, 3, 4, 5, 6, 7, or 8 inosine nucleosides. In some embodiments, X1 and X2 are each 12 nucleotides and comprise at least 10%, 20%, 30% or 40% inosine nucleosides.

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ comprises a sequence motif $[AUCN_5]_x$ , wherein $N_5$ is comprises guanosine or inosine, wherein x is an integer whose value indicates the number of sequence motifs, and wherein x= 3 or 4;

(viii) $X_2$ comprises a sequence motif $[CN_6AU]_y$, wherein N6 comprises guanosine or inosine, wherein y is an integer whose value indicates the number of sequence motifs, and wherein y = 3 or 4;

(ix) L is a linker that operably links the first polynucleotide and the second polynucleotide,

optionally, wherein at least one of N1, N2, N3, and N4 is inosine, and wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA. In some embodiments, N5 comprises inosine and N6 comprises inosine. In some embodiments, N5 comprises guanosine and N6 comprises inosine. In some embodiments, N5 comprises inosine and N6 comprises guanosine. In some embodiments, N5 comprises guanosine (G) and N6 comprises guanosine (G). In some

embodiments, x = 3 and y = 3. In some embodiments, x = 4 and y = 4. In some embodiments, N1 comprises inosine (I) and N4 comprises cytidine (C). In some embodiments, N2 comprises inosine (I) and N3 comprises cytidine (C). In some embodiments, N3 comprises inosine (I) and N2 comprises cytidine (C). In some embodiments, N4 comprises inosine (I) and N1 comprises cytidine (C). In some embodiments, N1 comprises guanosine (G). In some embodiments, N2 comprises guanosine (G). In some embodiments, N1 comprises cytidine (C). In some embodiments, N2 comprises cytidine (C). In some embodiments, N1 and N2 comprise guanosine (G) and N3 and N4 comprise cytidine (C). In some embodiments, N1 and N2 comprise cytidine (C) and N3 and N4 comprise guanosine (G). In some embodiments, N1 and N2 comprise inosine (I) and N3 and N4 comprise cytidine (C). In some embodiments, N1 and N2 comprise cytidine (C) and N3 and N4 comprise inosine (I).

In some embodiments, the linker (L) is a nucleotide linker or a non-nucleotide linker. In some embodiments, the linker (L) is a nucleotide linker comprising a tetraloop, wherein the nucleotide sequence of the tetraloop is selected from the group consisting of:

(a) UNCG, wherein N = A, C, G, or U;

(b) GNRA, wherein N = A, C, G, or U, and wherein R = A or G;

(c) ANYA, wherein N = A, C, G, or U, and wherein Y = C or T;

(d) CUYG, wherein Y = C or T;

(e) UMAC, wherein M = A or C; and

(f) CUUG.

In some embodiments, the linker (L) is a nucleotide linker comprising the nucleotide sequence UUUGAU or UGUUU. In some embodiments, the nucleotide linker comprises the nucleotide sequence UUUGAU. In some embodiments, the nucleotide linker comprises the nucleotide sequence UGUUU.

In some embodiments, the linker (L) is a nucleotide linker comprising a tetraloop, wherein the sequence of the tetraloop is UUCG. In some embodiments, the sequence of the tetraloop is GAUC.

In some embodiments, the linker (L) is a non-nucleotide linker selected from the group consisting of:

(a) an ethylene glycol linker; and

(b) an alkyl linker.

In some embodiments, the non-nucleotide linker is a hexaethylene glycol linker. In some embodiments, the non-nucleotide linker is a C9 alkyl linker.

In some embodiments, the RLR agonist comprises a 5' diphosphate moiety, or a derivative or analog thereof. In some embodiments, the agonist comprises a 5' triphosphate moiety, or a derivative or analog thereof. In some embodiments, the derivative or analog thereof comprises a phosphate bioisostere is selected from: a phosphonate, a thiophosphonate, a phosphorothioate, a sulfate, a sulfonate, a sulfamate, a thiazolidinone, a carboxylate, a malonate, a boronic acid, a benzoxaborole, a boranophosphate, a squaramide.

In some embodiments, the RLR agonist comprises a modified nucleotide, a modified nucleoside, or a modified nucleobase, or a combination thereof. In some embodiments, the agonist comprises a modification to the internucleotide linkages or to the polynucleotide backbone.

In some embodiments, the RLR agonist exhibits at least one or more of the following properties:

(a) specifically binds to one or more RLRs (e.g. RIG-1, MDA5 and/or LGP2);

(b) increases RLR-mediated cytokine production;

(c) increases RLR-mediated expression of interferon-stimulated genes (ISGs);

(d) increases RLR-dependent intracellular signaling;

(e) increases stability of the duplex;

(f) increases binding affinity to RLRs;

(g) decreases off-target binding;

(h) increases biological half-life;

(i) increases biodistribution and bioavailability;

(j) increases and/or enhances uptake into cells and/or tissues;

(k) decreases immunogenicity; and

(l) a combination of any of (a)-(k).

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first polynucleotide comprises a 5' diphosphate or triphosphate moiety, or

derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 and 36.

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first polynucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively.

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 22, 23 and 25.

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to a RIG-I-like receptor (RLR), wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 58 and 89, respectively;

(ii) SEQ ID NO: 59 and 89, respectively; and

(iii) SEQ ID NO: 61 and 91, respectively.

In some aspects, the disclosure provides a synthetic RIG-I-like receptor (RLR) agonist that specifically binds to RIG-I-like receptors (RLRs), wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively.

In some aspects, the disclosure provides RLR agonists wherein the nucleotide sequence comprising the agonist is not complementary to a genomic DNA sequence or mRNA sequence, wherein the RLR agonist does not participate in RNA interference, and wherein the RLR agonist does not silence gene expression.

**RLR Agonists Comprising Modified Nucleobases, Nucleosides, or Nucleotides**

In some embodiments, an RLR agonist of the disclosure comprises one or more modified nucleobases, nucleosides, or nucleotides. In some embodiments, modified RLR agonists may have useful properties, including enhanced stability, intracellular retention, enhanced target binding, and/or an increase in induction of the innate immune response of a cell into which the RLR agonist is introduced, as compared to a reference unmodified RLR agonist. Therefore, use of modified RLR agonists may enhance the efficiency of target binding, intracellular retention of nucleic acids, as well as possess reduced immunogenicity. In one embodiment, the agonist provided by the disclosure is comprised of one or more oligonucleotides that comprise at least one region modified to increase target binding affinity. Affinity of an oligonucleotide for its target polypeptide (e.g. an RLR receptor) can be determined by, for example, measuring the degree of fluorescence polarization (FP) upon binding of a fluorescently-labeled oligonucleotide to its target (Moerke (2009) Curr Protoc Chem Biol 1(1):1-15).

In another embodiment, the RLR agonist provided by the disclosure is comprised of at least one oligonucleotide comprising at least one region comprising at least one modified nucleobase, nucleoside, or nucleotide that increases the stability of the duplex. The stability of the duplex can be routinely determined by measuring the Tm of the duplex, which is the temperature at which the two oligonucleotide strands comprising the duplex dissociate; dissociation is detected spectrophotometrically. The higher the Tm, the greater the stability of the duplex.

In one embodiment, the region of the oligonucleotide which is modified to increase duplex stability comprises at least one nucleotide modified at the 2′ position of the sugar, most preferably a 2′-O-alkyl, 2′-O-alkyl-O-alkyl or 2′-fluoro-modified nucleotide. In another embodiment, an oligonucleotide comprising an RLR agonist is also modified to enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than an unmodified oligonucleotide. Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel electrophoresis. Oligonucleotides which have been modified to enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. In one embodiment, oligonucleotides which contain at least one phosphorothioate modification are used. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance (De Mesmaeker et al., 1995, Acc. Chem. Res. 28:366-374).

Specific examples of some oligonucleotides envisioned for this invention include those containing modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. In some embodiments, oligonucleotides with phosphorothioate backbones (including those synthesized in a stereo-specific manner) and those with heteroatom backbones, particularly CH2—NH—O—CH2, CH2—N(CH3)—O—CH2 [known as a methylene(methylimino) or MMI backbone], CH2—O—N(CH3)—CH2, CH2—N(CH3)—N(CH3)—CH2 and O—N(CH3)—CH2—CH2 backbones, wherein the native phosphodiester backbone is represented as O—P—O—CH2) are used. The amide backbones disclosed by De Mesmaeker et al. (1995, Acc. Chem. Res. 28:366-374) are also used in some embodiments. Oligonucleotides may also contain one or more substituted sugar moieties. In some embodiments, oligonucleotides comprise one of the following at the 2′ position: OH, SH, SCH3, F, OCN, OCH3OCH3, OCH3O(CH2)nCH3, O(CH2)nNH2 or O(CH2)nCH3 where n is from 1 to about 10; C1 to C10 lower alkyl, alkoxyalkoxy (also known in the art as O-alkyl-O-alkyl), substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF3; OCF3; O—, S—, or N-alkyl; O—, S—, or N-

alkenyl; SOCH3; SO2CH3; ONO2; NO2; N3; NH2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. In one embodiment, a modification includes 2′-methoxyethoxy [2′—O—CH2CH2OCH3, also known as 2′—O—(2-methoxyethyl) or 2′-MOE] (Martin et al., Helv. Chim. Acta, 1995, 78, 486). In some embodiments, modifications include 2′-methoxy (2′—O—CH3), 2′-propoxy (2′—OCH2CH2CH3) and 2′-fluoro (2′-F) . Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3′ position of the sugar on the 3′ terminal nucleotide and the 5′ position of 5′ terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Oligonucleotides may also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2′deoxycytosine and often referred to in the art as 5-me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N6(6-aminohexyl)adenine and 2,6-diaminopurine. Kornberg, A., DNA Replication, W.H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu, G., et al., 1987, Nucl. Acids Res. 15:4513). A "universal" base known in the art, e.g., inosine, may be included. 5-me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently used in some embodiments as base substitutions.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553), cholic acid (Manoharan et al., Bioorg. Med. Chem. Let., 1994, 4, 1053), a thioether, e.g.,

hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 111; Kabanov et al., FEBS Lett., 1990, 259, 327; Svinarchuk et al., Biochimie, 1993, 75, 49), a phospholipid, a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S. Pat. Nos. 5,138,045, 5,218,105 and 5,459,255.

The oligonucleotides of the invention may be provided as prodrugs, which comprise one or more moieties which are cleaved off, generally in the body, to yield an active oligonucleotide. One example of a prodrug approach is described by Imbach et al. in WO Publication 94/26764.

It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a single nucleoside within an oligonucleotide.

The oligonucleotides in accordance with this invention preferably are from about 8 to about 50 nucleotides in length. In the context of this invention it is understood that this encompasses non-naturally occurring oligomers as hereinbefore described, having 8 to 50 monomers.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the knowledge and ability of one of ordinary skill in the art. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives. It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling Va.) to synthesize fluorescently labeled, biotinylated or other modified oligonucleotides such as cholesterol-modified oligonucleotides.

In some embodiments, an RLR agonist includes one or more (e.g., 1, 2, 3 or 4) different modified nucleobases, nucleosides, or nucleotides. In some embodiments, an RLR agonist

includes one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or more) different modified nucleobases, nucleosides, or nucleotides. In some embodiments, the modified RLR agonist may have reduced degradation in a cell into which the RLR agonist is introduced, relative to a corresponding unmodified RLR agonist.

In some embodiments, the modified nucleobase is a modified uracil. Exemplary nucleobases and nucleosides having a modified uracil include pseudouridine (ψ), pyridin-4-one ribonucleoside, 5-aza-uridine, 6-aza-uridine, 2-thio-5-aza-uridine, 2-thio-uridine ($s^2U$), 4-thio-uridine ($s^4U$), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine ($ho^5U$), 5-aminoallyl-uridine, 5-halo-uridine (e.g., 5-iodo-uridineor 5-bromo-uridine), 3-methyl-uridine ($m^3U$), 5-methoxy-uridine ($mo^5U$), uridine 5-oxyacetic acid ($cmo^5U$), uridine 5-oxyacetic acid methyl ester ($mcmo^5U$), 5-carboxymethyl-uridine ($cm^5U$), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uridine ($chm^5U$), 5-carboxyhydroxymethyl-uridine methyl ester ($mchm^5U$), 5-methoxycarbonylmethyl-uridine ($mcm^5U$), 5-methoxycarbonylmethyl-2-thio-uridine ($mcm^5s^2U$), 5-aminomethyl-2-thio-uridine ($nm^5s^2U$), 5-methylaminomethyl-uridine ($mnm^5U$), 5-methylaminomethyl-2-thio-uridine ($mnm^5s^2U$), 5-methylaminomethyl-2-seleno-uridine ($mnm^5se^2U$), 5-carbamoylmethyl-uridine ($ncm^5U$), 5-carboxymethylaminomethyl-2-thio-uridine ($cmnm^5s^2U$), 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyl-uridine ($\tau m^5U$), 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine($\tau m^5s^2U$), 1-taurinomethyl-4-thio-pseudouridine, 5-methyl-uridine ($m^5U$, i.e., having the nucleobase deoxythymine), 1-methyl-pseudouridine ($m^1\psi$), 5-methyl-2-thio-uridine ($m^5s^2U$), 1-methyl-4-thio-pseudouridine ($m^1s^4\psi$), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine ($m^3\psi$), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine (D), dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-dihydrouridine ($m^5D$), 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxy-uridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 3-(3-amino-3-carboxypropyl)uridine ($acp^3U$), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($acp^3\psi$), 5-(isopentenylaminomethyl)uridine ($inm^5U$), 5-(isopentenylaminomethyl)-2-thio-uridine ($inm^5s^2U$), α-thio-uridine, 2′-O-methyl-uridine (Um), 5,2′-O-dimethyl-uridine ($m^5Um$), 2′-O-methyl-pseudouridine (ψm), 2-thio-2′-O-methyl-uridine ($s^2Um$), 5-methoxycarbonylmethyl-2′-O-methyl-uridine ($mcm^5Um$), 5-carbamoylmethyl-2′-O-methyl-uridine ($ncm^5Um$), 5-

carboxymethylaminomethyl-2′-O-methyl-uridine (cmnm$^5$Um), 3,2′-O-dimethyl-uridine (m$^3$Um), and 5-(isopentenylaminomethyl)-2′-O-methyl-uridine (inm$^5$Um), 1-thio-uridine, deoxythymidine, 2'-F-ara-uridine, 2'-F-uridine, 2'-OH-ara-uridine, 5-(2-carbomethoxyvinyl) uridine, and 5-[3-(1-E-propenylamino)]uridine.

In some embodiments, the modified nucleobase is a modified cytosine. Exemplary nucleobases and nucleosides having a modified cytosine include 5-aza-cytidine, 6-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine (m$^3$C), N4-acetyl-cytidine (ac$^4$C), 5-formyl-cytidine (f$^5$C), N4-methyl-cytidine (m$^4$C), 5-methyl-cytidine (m$^5$C), 5-halo-cytidine (e.g., 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm$^5$C), 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine (s$^2$C), 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, lysidine (k$_2$C), α-thio-cytidine, 2′-O-methyl-cytidine (Cm), 5,2′-O-dimethyl-cytidine (m$^5$Cm), N4-acetyl-2′-O-methyl-cytidine (ac$^4$Cm), N4,2′-O-dimethyl-cytidine (m$^4$Cm), 5-formyl-2′-O-methyl-cytidine (f$^5$Cm), N4,N4,2′-O-trimethyl-cytidine (m$^4{}_2$Cm), 1-thio-cytidine, 2'-F-ara-cytidine, 2'-F-cytidine, and 2'-OH-ara-cytidine.

In some embodiments, the modified nucleobase is a modified adenine. Exemplary nucleobases and nucleosides having a modified adenine include α-thio-adenosine, 2-amino-purine, 2, 6-diaminopurine, 2-amino-6-halo-purine (e.g., 2-amino-6-chloro-purine), 6-halo-purine (e.g., 6-chloro-purine), 2-amino-6-methyl-purine, 8-azido-adenosine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-amino-purine, 7-deaza-8-aza-2-amino-purine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyl-adenosine (m$^1$A), 2-methyl-adenine (m$^2$A), N6-methyl-adenosine (m$^6$A), 2-methylthio-N6-methyl-adenosine (ms$^2$m$^6$A), N6-isopentenyl-adenosine (i$^6$A), 2-methylthio-N6-isopentenyl-adenosine (ms$^2$i$^6$A), N6-(cis-hydroxyisopentenyl)adenosine (io$^6$A), 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine (ms$^2$io$^6$A), N6-glycinylcarbamoyl-adenosine (g$^6$A), N6-threonylcarbamoyl-adenosine (t$^6$A), N6-methyl-N6-threonylcarbamoyl-adenosine (m$^6$t$^6$A), 2-methylthio-N6-threonylcarbamoyl-adenosine (ms$^2$g$^6$A), N6,N6-dimethyl-adenosine (m$^6{}_2$A), N6-hydroxynorvalylcarbamoyl-adenosine (hn$^6$A), 2-methylthio-N6-hydroxynorvalylcarbamoyl-adenosine (ms$^2$hn$^6$A), N6-acetyl-

adenosine ($ac^6A$), 7-methyl-adenine, 2-methylthio-adenine, 2-methoxy-adenine, α-thio-adenosine, 2′-O-methyl-adenosine (Am), N6,2′-O-dimethyl-adenosine ($m^6Am$), N6,N6,2′-O-trimethyl-adenosine ($m^6{}_2Am$), 1,2′-O-dimethyl-adenosine ($m^1Am$), 2′-O-ribosyladenosine (phosphate) (Ar(p)), 2-amino-N6-methyl-purine, 1-thio-adenosine, 8-azido-adenosine, 2'-F-ara-adenosine, 2'-F-adenosine, 2'-OH-ara-adenosine, and N6-(19-amino-pentaoxanonadecyl)-adenosine.

In some embodiments, the modified nucleobase is a modified guanine. Exemplary nucleobases and nucleosides having a modified guanine include α-thio-guanosine, inosine (I), 1-methyl-inosine ($m^1I$), wyosine (imG), methylwyosine (mimG), 4-demethyl-wyosine (imG-14), isowyosine (imG2), wybutosine (yW), peroxywybutosine ($o_2yW$), hydroxywybutosine (OhyW), undermodified hydroxywybutosine (OhyW*), 7-deaza-guanosine, queuosine (Q), epoxyqueuosine (oQ), galactosyl-queuosine (galQ), mannosyl-queuosine (manQ), 7-cyano-7-deaza-guanosine ($preQ_0$), 7-aminomethyl-7-deaza-guanosine ($preQ_1$), archaeosine ($G^+$), 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine ($m^7G$), 6-thio-7-methyl-guanosine, 7-methyl-inosine, 6-methoxy-guanosine, 1-methyl-guanosine ($m^1G$), N2-methyl-guanosine ($m^2G$), N2,N2-dimethyl-guanosine ($m^2{}_2G$), N2,7-dimethyl-guanosine ($m^{2,7}G$), N2, N2,7-dimethyl-guanosine ($m^{2,2,7}G$), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, N2,N2-dimethyl-6-thio-guanosine, α-thio-guanosine, 2′-O-methyl-guanosine (Gm), N2-methyl-2′-O-methyl-guanosine ($m^2Gm$), N2,N2-dimethyl-2′-O-methyl-guanosine ($m^2{}_2Gm$), 1-methyl-2′-O-methyl-guanosine ($m^1Gm$), N2,7-dimethyl-2′-O-methyl-guanosine ($m^{2,7}Gm$), 2′-O-methyl-inosine (Im), 1,2′-O-dimethyl-inosine ($m^1Im$), 2′-O-ribosylguanosine (phosphate) (Gr(p)) , 1-thio-guanosine, O6-methyl-guanosine, 2'-F-ara-guanosine, and 2'-F-guanosine.

In some embodiments, an RLR agonist of the disclosure includes a combination of one or more of the aforementioned modified nucleobases (e.g., a combination of 2, 3 or 4 of the aforementioned modified nucleobases.)

In certain embodiments, an RLR agonist of the disclosure is uniformly modified (i.e., fully modified, modified through-out the entire sequence) for a particular modification. For example, an RLR agonist can be uniformly modified with 5-methyl-cytidine ($m^5C$), meaning that all cytosine residues in the mRNA sequence are replaced with 5-methyl-cytidine ($m^5C$). Similarly, an RLR agonist of the disclosure can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

Examples of nucleoside modifications and combinations thereof that may be present in an RLR agonist of the present disclosure include, but are not limited to, those described in PCT Patent Application Publications: WO2012045075, WO2014081507, WO2014093924, WO2014164253, and WO2014159813.

The RLR agonists of the disclosure can include a combination of modifications to the sugar, the nucleobase, and/or the internucleoside linkage. These combinations can include any one or more modifications described herein.

Examples of modified nucleosides and modified nucleoside combinations are provided below in Table 1 and Table 2. These combinations of modified nucleotides can be used to form the RLR agonists of the disclosure. In certain embodiments, the modified nucleosides may be partially or completely substituted for the natural nucleotides of the RLR agonists of the disclosure. As a non-limiting example, the natural nucleotide uridine may be substituted with a modified nucleoside described herein. In another non-limiting example, the natural nucleoside uridine may be partially substituted (e.g., about 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99.9% of the natural uridines) with at least one of the modified nucleoside disclosed herein.

**Table 1. Combinations of Nucleoside Modifications**

| Modified Nucleotide | Modified Nucleotide Combination |
|---|---|
| α-thio-cytidine | α-thio-cytidine/5-iodo-uridine |
| | α-thio-cytidine/N1-methyl-pseudouridine |
| | α-thio-cytidine/α-thio-uridine |
| | α-thio-cytidine/5-methyl-uridine |
| | α-thio-cytidine/pseudo-uridine |
| | about 50% of the cytosines are α-thio-cytidine |
| pseudoisocytidine | pseudoisocytidine/5-iodo-uridine |
| | pseudoisocytidine/N1-methyl-pseudouridine |
| | pseudoisocytidine/α-thio-uridine |
| | pseudoisocytidine/5-methyl-uridine |
| | pseudoisocytidine/pseudouridine |
| | about 25% of cytosines are pseudoisocytidine |

| | |
|---|---|
| | pseudoisocytidine/about 50% of uridines are N1-methyl-pseudouridine and about 50% of uridines are pseudouridine |
| | pseudoisocytidine/about 25% of uridines are N1-methyl-pseudouridine and about 25% of uridines are pseudouridine |
| pyrrolo-cytidine | pyrrolo-cytidine/5-iodo-uridine |
| | pyrrolo-cytidine/N1-methyl-pseudouridine |
| | pyrrolo-cytidine/α-thio-uridine |
| | pyrrolo-cytidine/5-methyl-uridine |
| | pyrrolo-cytidine/pseudouridine |
| | about 50% of the cytosines are pyrrolo-cytidine |
| 5-methyl-cytidine | 5-methyl-cytidine/5-iodo-uridine |
| | 5-methyl-cytidine/N1-methyl-pseudouridine |
| | 5-methyl-cytidine/α-thio-uridine |
| | 5-methyl-cytidine/5-methyl-uridine |
| | 5-methyl-cytidine/pseudouridine |
| | about 25% of cytosines are 5-methyl-cytidine |
| | about 50% of cytosines are 5-methyl-cytidine |
| | 5-methyl-cytidine/5-methoxy-uridine |
| | 5-methyl-cytidine/5-bromo-uridine |
| | 5-methyl-cytidine/2-thio-uridine |
| | 5-methyl-cytidine/about 50% of uridines are 2-thio-uridine |
| | about 50% of uridines are 5-methyl-cytidine/ about 50% of uridines are 2-thio-uridine |
| N4-acetyl-cytidine | N4-acetyl-cytidine /5-iodo-uridine |
| | N4-acetyl-cytidine /N1-methyl-pseudouridine |
| | N4-acetyl-cytidine /α-thio-uridine |
| | N4-acetyl-cytidine /5-methyl-uridine |
| | N4-acetyl-cytidine /pseudouridine |
| | about 50% of cytosines are N4-acetyl-cytidine |
| | about 25% of cytosines are N4-acetyl-cytidine |
| | N4-acetyl-cytidine /5-methoxy-uridine |
| | N4-acetyl-cytidine /5-bromo-uridine |
| | N4-acetyl-cytidine /2-thio-uridine |

| | |
|---|---|
| | about 50% of cytosines are N4-acetyl-cytidine/ about 50% of uridines are 2-thio-uridine |

**Table 2. Modified Nucleosides and Combinations Thereof**

| |
|---|
| 1-(2,2,2-Trifluoroethyl)pseudo-UTP |
| 1-Ethyl-pseudo-UTP |
| 1-Methyl-pseudo-U-alpha-thio-TP |
| 1-methyl-pseudouridine TP, ATP, GTP, CTP |
| 1-methyl-pseudo-UTP/5-methyl-CTP/ATP/GTP |
| 1-methyl-pseudo-UTP/CTP/ATP/GTP |
| 1-Propyl-pseudo-UTP |
| 25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Aminoallyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Bromo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Bromo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Bromo-CTP + 75 % CTP/1-Methyl-pseudo-UTP |
| 25 % 5-Carboxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Carboxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Ethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Ethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Ethynyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Ethynyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Fluoro-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Fluoro-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Formyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Formyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Hydroxymethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Hydroxymethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Iodo-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |

| |
|---|
| 25 % 5-Iodo-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Methoxy-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Methoxy-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/50 % 5-Methoxy-UTP + 50 % UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/5-Methoxy-UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP |
| 25 % 5-Methyl-CTP + 75 % CTP/75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Phenyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Phenyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Trifluoromethyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % 5-Trifluoromethyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % 5-Trifluoromethyl-CTP + 75 % CTP/1-Methyl-pseudo-UTP |
| 25 % N4-Ac-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % N4-Ac-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % N4-Bz-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % N4-Bz-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % N4-Methyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % N4-Methyl-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25 % Pseudo-iso-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 25 % Pseudo-iso-CTP + 75 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 25% 5-Bromo-CTP/75% CTP/ Pseudo-UTP |
| 25% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP |

| |
|---|
| 25% 5-methoxy-UTP/5-methyl-CTP/ATP/GTP |
| 25% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP |
| 25% 5-methoxy-UTP/CTP/ATP/GTP |
| 25% 5-metoxy-UTP/50% 5-methyl-CTP/ATP/GTP |
| 2-Amino-ATP |
| 2-Thio-CTP |
| 2-thio-pseudouridine TP, ATP, GTP, CTP |
| 2-Thio-pseudo-UTP |
| 2-Thio-UTP |
| 3-Methyl-CTP |
| 3-Methyl-pseudo-UTP |
| 4-Thio-UTP |
| 50 % 5-Bromo-CTP + 50 % CTP/1-Methyl-pseudo-UTP |
| 50 % 5-Hydroxymethyl-CTP + 50 % CTP/1-Methyl-pseudo-UTP |
| 50 % 5-methoxy-UTP/5-methyl-CTP/ATP/GTP |
| 50 % 5-Methyl-CTP + 50 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP |
| 50 % 5-Methyl-CTP + 50 % CTP/25 % 5-Methoxy-UTP + 75 % UTP |
| 50 % 5-Methyl-CTP + 50 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP |
| 50 % 5-Methyl-CTP + 50 % CTP/50 % 5-Methoxy-UTP + 50 % UTP |
| 50 % 5-Methyl-CTP + 50 % CTP/5-Methoxy-UTP |
| 50 % 5-Methyl-CTP + 50 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP |
| 50 % 5-Methyl-CTP + 50 % CTP/75 % 5-Methoxy-UTP + 25 % UTP |
| 50 % 5-Trifluoromethyl-CTP + 50 % CTP/1-Methyl-pseudo-UTP |
| 50% 5-Bromo-CTP/ 50% CTP/Pseudo-UTP |
| 50% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP |
| 50% 5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP |
| 50% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP |

| |
|---|
| 50% 5-methoxy-UTP/CTP/ATP/GTP |
| 5-Aminoallyl-CTP |
| 5-Aminoallyl-CTP/ 5-Methoxy-UTP |
| 5-Aminoallyl-UTP |
| 5-Bromo-CTP |
| 5-Bromo-CTP/ 5-Methoxy-UTP |
| 5-Bromo-CTP/1-Methyl-pseudo-UTP |
| 5-Bromo-CTP/Pseudo-UTP |
| 5-bromocytidine TP, ATP, GTP, UTP |
| 5-Bromo-UTP |
| 5-Carboxy-CTP/ 5-Methoxy-UTP |
| 5-Ethyl-CTP/5-Methoxy-UTP |
| 5-Ethynyl-CTP/5-Methoxy-UTP |
| 5-Fluoro-CTP/ 5-Methoxy-UTP |
| 5-Formyl-CTP/ 5-Methoxy-UTP |
| 5-Hydroxy- methyl-CTP/ 5-Methoxy-UTP |
| 5-Hydroxymethyl-CTP |
| 5-Hydroxymethyl-CTP/1-Methyl-pseudo-UTP |
| 5-Hydroxymethyl-CTP/5-Methoxy-UTP |
| 5-hydroxymethyl-cytidine TP, ATP, GTP, UTP |
| 5-Iodo-CTP/ 5-Methoxy-UTP |
| 5-Me-CTP/5-Methoxy-UTP |
| 5-Methoxy carbonyl methyl-UTP |
| 5-Methoxy-CTP/5-Methoxy-UTP |
| 5-methoxy-uridine TP, ATP, GTP, UTP |
| 5-methoxy-UTP |
| 5-Methoxy-UTP |
| 5-Methoxy-UTP/ N6-Isopentenyl-ATP |
| 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP |
| 5-methoxy-UTP/5-methyl-CTP/ATP/GTP |

| |
|---|
| 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP |
| 5-methoxy-UTP/CTP/ATP/GTP |
| 5-Methyl-2-thio-UTP |
| 5-Methylaminomethyl-UTP |
| 5-Methyl-CTP/ 5-Methoxy-UTP |
| 5-Methyl-CTP/ 5-Methoxy-UTP(cap 0) |
| 5-Methyl-CTP/ 5-Methoxy-UTP(No cap) |
| 5-Methyl-CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP |
| 5-Methyl-CTP/25 % 5-Methoxy-UTP + 75 % UTP |
| 5-Methyl-CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP |
| 5-Methyl-CTP/50 % 5-Methoxy-UTP + 50 % UTP |
| 5-Methyl-CTP/5-Methoxy-UTP/N6-Me-ATP |
| 5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP |
| 5-Methyl-CTP/75 % 5-Methoxy-UTP + 25 % UTP |
| 5-Phenyl-CTP/ 5-Methoxy-UTP |
| 5-Trifluoro- methyl-CTP/ 5-Methoxy-UTP |
| 5-Trifluoromethyl-CTP |
| 5-Trifluoromethyl-CTP/ 5-Methoxy-UTP |
| 5-Trifluoromethyl-CTP/1-Methyl-pseudo-UTP |
| 5-Trifluoromethyl-CTP/Pseudo-UTP |
| 5-Trifluoromethyl-UTP |
| 5-trifluromethylcytidine TP, ATP, GTP, UTP |
| 75 % 5-Aminoallyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Aminoallyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Bromo-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Bromo-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Carboxy-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Carboxy-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Ethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Ethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |

| |
|---|
| 75 % 5-Ethynyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Ethynyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Fluoro-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Fluoro-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Formyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Formyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Hydroxymethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Hydroxymethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Iodo-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Iodo-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Methoxy-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Methoxy-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-methoxy-UTP/5-methyl-CTP/ATP/GTP |
| 75 % 5-Methyl-CTP + 25 % CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP |
| 75 % 5-Methyl-CTP + 25 % CTP/25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Methyl-CTP + 25 % CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP |
| 75 % 5-Methyl-CTP + 25 % CTP/50 % 5-Methoxy-UTP + 50 % UTP |
| 75 % 5-Methyl-CTP + 25 % CTP/5-Methoxy-UTP |
| 75 % 5-Methyl-CTP + 25 % CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP |
| 75 % 5-Methyl-CTP + 25 % CTP/75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Phenyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % 5-Phenyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Trifluoromethyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |

| |
|---|
| 75 % 5-Trifluoromethyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % 5-Trifluoromethyl-CTP + 25 % CTP/1-Methyl-pseudo-UTP |
| 75 % N4-Ac-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % N4-Ac-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % N4-Bz-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % N4-Bz-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % N4-Methyl-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % N4-Methyl-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75 % Pseudo-iso-CTP + 25 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP |
| 75 % Pseudo-iso-CTP + 25 % CTP/ 75 % 5-Methoxy-UTP + 25 % UTP |
| 75% 5-Bromo-CTP/25% CTP/ 1-Methyl-pseudo-UTP |
| 75% 5-Bromo-CTP/25% CTP/ Pseudo-UTP |
| 75% 5-methoxy-UTP/25% 5-methyl-CTP/ATP/GTP |
| 75% 5-methoxy-UTP/50% 5-methyl-CTP/ATP/GTP |
| 75% 5-methoxy-UTP/75% 5-methyl-CTP/ATP/GTP |
| 75% 5-methoxy-UTP/CTP/ATP/GTP |
| 8-Aza-ATP |
| Alpha-thio-CTP |
| CTP/25 % 5-Methoxy-UTP + 75 % 1-Methyl-pseudo-UTP |
| CTP/25 % 5-Methoxy-UTP + 75 % UTP |
| CTP/50 % 5-Methoxy-UTP + 50 % 1-Methyl-pseudo-UTP |
| CTP/50 % 5-Methoxy-UTP + 50 % UTP |
| CTP/5-Methoxy-UTP |
| CTP/5-Methoxy-UTP (cap 0) |
| CTP/5-Methoxy-UTP(No cap) |
| CTP/75 % 5-Methoxy-UTP + 25 % 1-Methyl-pseudo-UTP |
| CTP/75 % 5-Methoxy-UTP + 25 % UTP |
| CTP/UTP(No cap) |
| N1-Me-GTP |

| |
|---|
| N4-Ac-CTP |
| N4Ac-CTP/1-Methyl-pseudo-UTP |
| N4Ac-CTP/5-Methoxy-UTP |
| N4-acetyl-cytidine TP, ATP, GTP, UTP |
| N4-Bz-CTP/ 5-Methoxy-UTP |
| N4-methyl CTP |
| N4-Methyl-CTP/ 5-Methoxy-UTP |
| Pseudo-iso-CTP/ 5-Methoxy-UTP |
| PseudoU-alpha-thio-TP |
| pseudouridine TP, ATP, GTP, CTP |
| pseudo-UTP/5-methyl-CTP/ATP/GTP |
| UTP-5-oxyacetic acid Me ester |
| Xanthosine |

According to the disclosure, polynucleotides of the disclosure may be synthesized to comprise the combinations or single modifications of Table 1 or Table 2.

Where a single modification is listed, the listed nucleoside or nucleotide represents 100 percent of that A, U, G or C nucleotide or nucleoside having been modified. Where percentages are listed, these represent the percentage of that particular A, U, G or C nucleobase triphosphate of the total amount of A, U, G, or C triphosphate present. For example, the combination: 25 % 5-Aminoallyl-CTP + 75 % CTP/ 25 % 5-Methoxy-UTP + 75 % UTP refers to a polynucleotide where 25% of the cytosine triphosphates are 5-Aminoallyl-CTP while 75% of the cytosines are CTP; whereas 25% of the uracils are 5-methoxy UTP while 75% of the uracils are UTP. Where no modified UTP is listed then the naturally occurring ATP, UTP, GTP and/or CTP is used at 100% of the sites of those nucleotides found in the polynucleotide. In this example all of the GTP and ATP nucleotides are left unmodified.

**Methods of Making RLR Agonists**

RLR agonists of the present disclosure may be produced by means available in the art, including but not limited to in vitro transcription (IVT) and synthetic methods. Enzymatic (IVT),

solid-phase, liquid-phase, combined synthetic methods, small region synthesis, and ligation methods may be utilized. In one embodiment, RLR agonists are made using IVT enzymatic synthesis methods. Methods of making polynucleotides by IVT are known in the art and are described in International Application PCT/US2013/30062, the contents of which are incorporated herein by reference in their entirety. Accordingly, the present disclosure also includes polynucleotides, e.g., DNA, constructs and vectors that may be used to in vitro transcribe an RLR agonist described herein.

Non-natural modified nucleobases may be introduced into polynucleotides, e.g., RNA, during synthesis or post-synthesis. In certain embodiments, modifications may be on internucleoside linkages, purine or pyrimidine bases, or sugar. In particular embodiments, the modification may be introduced at the terminal of a polynucleotide chain or anywhere else in the polynucleotide chain; with chemical synthesis or with a polymerase enzyme. Examples of modified nucleic acids and their synthesis are disclosed in PCT application No. PCT/US2012/058519. Synthesis of modified polynucleotides is also described in Verma and Eckstein, Annual Review of Biochemistry, vol. 76, 99-134 (1998).

Either enzymatic or chemical ligation methods may be used to conjugate polynucleotides or their regions with different functional moieties, such as targeting or delivery agents, fluorescent labels, liquids, nanoparticles, etc. Conjugates of polynucleotides and modified polynucleotides are reviewed in Goodchild, Bioconjugate Chemistry, vol. 1(3), 165-187 (1990).

The synthesis of oligonucleotides, polynucleotides, and conjugations and ligations thereof, is further described in Taskova et al., (2017) Chembiochem 18(17):1671-1682; Gooding et al., (2016) Eur J Pharm Biopharm 107:321-40; Menzi et al., (2015) Future Med Chem 7(13):1733-49; Winkler J., (2013) Ther Deliv. (7):791-809; Singh et al., (2010) Chem Soc Rev 39(6):2054-70; and Lu et al., (2010) Bioconjug Chem 21(2):187-202.

**Virus-like Particles (VLPs)**

In some embodiments, the disclosure provides compositions comprising at least one RLR agonist described and a virus-like particle (VLP). In some embodiments, an RLR agonist is bound to the VLP. In some embodiments, an RLR agonist is packaged in the VLP.

Virus-like particles in the context of the present application refer to structures resembling a virus particle, but which are not pathogenic. In general, virus-like particles lack the viral genome

and, therefore, are noninfectious. Also, virus-like particles can be produced in large quantities by heterologous expression and can be easily purified.

Exemplary virus-like particles suitable for use in the compositions described herein are provided in PCT Publication Nos. WO 2003/024481 and WO 2004/084940, each of which is herein incorporated in its entirety by this reference.

In some embodiments, the virus-like particle is a recombinant virus-like particle. The skilled artisan can produce VLPs using recombinant DNA technology and virus coding sequences which are readily available to the public and described herein. For example, the coding sequence of a virus envelope or core protein can be engineered for expression in a baculovirus expression vector using a commercially available baculovirus vector, under the regulatory control of a virus promoter, with appropriate modifications of the sequence to allow functional linkage of the coding sequence to the regulatory sequence. The coding sequence of a virus envelope or core protein can also be engineered for expression in a bacterial expression vector, for example.

Examples of VLPs include, but are not limited to, the capsid proteins of Hepatitis B virus (Ulrich, et al., Virus Res. 50:141-182 (1998)), measles virus (Warnes, et al., Gene 160:173-178 (1995)), Sindbis virus, rotavirus (U.S. Pat. Nos. 5,071,651 and 5,374,426), foot-and-mouth-disease virus (Twomey, et al., Vaccine 13:1603-1610, (1995)), Norwalk virus (Jiang, X., et al., Science 250:1580-1583 (1990); Matsui, S. M., et al., J. Clin. Invest. 87:1456-1461 (1991)), the retroviral GAG protein (PCT Patent Appl. No. WO 96/30523), the retrotransposon Ty protein p1, the surface protein of Hepatitis B virus (WO 92/11291), human papilloma virus (WO 98/15631), human polyoma virus (Sasnauskas K., et al., Biol. Chem. 380(3):381-386 (1999); Sasnauskas K., et al., Generation of recombinant virus-like particles of different polyomaviruses in yeast 3rd International Workshop "Virus-like particles as vaccines." Berlin, Sep. 26-29, 2001), RNA phages, Ty, fr-phage, GA-phage, AP 205-phage and, in particular, Qβ-phage.

As will be readily apparent to those skilled in the art, the VLP of the disclosure is not limited to any specific form. The particle can be synthesized chemically or through a biological process, which can be natural or non-natural. By way of example, this type of embodiment includes a virus-like particle or a recombinant form thereof. In some embodiments, the VLP comprises recombinant polypeptides of Rotavirus; recombinant polypeptides of Norwalk virus; recombinant polypeptides of Alphavirus; recombinant proteins which form bacterial pili or pilus-like structures; recombinant polypeptides of Foot and Mouth Disease virus; recombinant polypeptides of measles

virus, recombinant polypeptides of Sindbis virus, recombinant polypeptides of Retrovirus; recombinant polypeptides of Hepatitis B virus (e.g., a HBcAg); recombinant polypeptides of Tobacco mosaic virus; recombinant polypeptides of Flock House Virus; recombinant polypeptides of human Papillomavirus; recombinant polypeptides of Polyoma virus and, in particular, recombinant polypeptides of human Polyoma virus, and in particular recombinant polypeptides of BK virus; recombinant polypeptides of bacteriophages, recombinant polypeptides of RNA phages; recombinant polypeptides of Ty; recombinant polypeptides of fr-phage, recombinant polypeptides of GA-phage, recombinant polypeptides of AP 205-phage and, in particular, recombinant polypeptides of Qβ-phage. The virus-like particle can further comprise, or alternatively consist of, one or more fragments of such polypeptides, as well as variants of such polypeptides. Variants of polypeptides can share, for example, at least 80%, 85%, 90%, 95%, 97%, or 99% identity at the amino acid level with their wild-type counterparts.

In some embodiments, the virus-like particle comprises recombinant proteins, or fragments thereof, of a RNA-phage. In some embodiments, the RNA-phage is selected from the group consisting of a) bacteriophage Qβ; b) bacteriophage R17; c) bacteriophage fr; d) bacteriophage GA; e) bacteriophage SP; f) bacteriophage MS2; g) bacteriophage M11; h) bacteriophage MX1; i) bacteriophage NL95; k) bacteriophage f2; and 1) bacteriophage PP7.

In some embodiments, the virus-like particle comprises recombinant proteins, or fragments thereof, of the RNA-bacteriophage Qβ or of the RNA-bacteriophage fr. In some embodiments, the virus-like particle comprises recombinant proteins, or fragments thereof, of the RNA-bacteriophage Qβ.

In some embodiments, the recombinant proteins comprise coat proteins of RNA phages.

RNA-phage coat proteins forming capsids or VLPs, or fragments of the bacteriophage coat proteins compatible with self-assembly into a capsid or a VLP, are, therefore, further embodiments of the present disclosure. Bacteriophage Qβ coat proteins, for example, can be expressed recombinantly in *E. coli*. Further, upon such expression these proteins spontaneously form capsids. Additionally, these capsids form a structure with an inherent repetitive organization.

Examples of bacteriophage coat proteins which can be used to prepare compositions of the disclosure include the coat proteins of RNA bacteriophages such as bacteriophage Qβ (SEQ ID NO: 112; PIR Database, Accession No. VCBPQb referring to Qβ CP and SEQ ID NO: 113; Accession No. AAA16663 referring to Qβ A1 protein), bacteriophage R17 (SEQ ID NO: 114; PIR

Accession No. VCBPR7), bacteriophage fr (SEQ ID NO: 115; PIR Accession No. VCBPFR), bacteriophage GA (SEQ ID NO: 116; GenBank Accession No. NP-040754), bacteriophage SP (SEQ ID NO: 117; GenBank Accession No. CAA30374 referring to SP CP and SEQ ID NO: 118; Accession No. referring to SP A1 protein), bacteriophage MS2 (SEQ ID NO: 119; PIR Accession No. VCBPM2), bacteriophage M11 (SEQ ID NO: 120; GenBank Accession No. AAC06250), bacteriophage MX1 (SEQ ID NO: 121; GenBank Accession No. AAC14699), bacteriophage NL95 (SEQ ID NO: 122; GenBank Accession No. AAC14704), bacteriophage f2 (SEQ ID NO: 123; GenBank Accession No. P03611), bacteriophage PP7 (SEQ ID NO: 124). Furthermore, the A1 protein of bacteriophage Qβ or C-terminal truncated forms missing as much as 100, 150 or 180 amino acids from its C-terminus may be incorporated in a capsid assembly of Qβ coat proteins. Generally, the percentage of Qβ A1 protein relative to Qβ CP in the capsid assembly will be limited, in order to ensure capsid formation.

Qβ coat protein has also been found to self-assemble into capsids when expressed in *E. coli* (Kozlovska T M. et al., GENE 137: 133-137 (1993)). The obtained capsids or virus-like particles showed an icosahedral phage-like capsid structure with a diameter of 25 nm and T=3 quasi symmetry. Further, the crystal structure of phage Qβ has been solved. The capsid contains 180 copies of the coat protein, which are linked in covalent pentamers and hexamers by disulfide bridges (Golmohammadi, R. et al., Structure 4: 543-5554 (1996)) leading to a remarkable stability of the capsid of Qβ coat protein. Capsids or VLPs made from recombinant Qβ coat protein may contain, however, subunits not linked via disulfide links to other subunits within the capsid, or incompletely linked. Thus, upon loading recombinant Qβ capsid on non-reducing SDS-PAGE, bands corresponding to monomeric Qβ coat protein as well as bands corresponding to the hexamer or pentamer of Qβ coat protein are visible. Incompletely disulfide-linked subunits could appear as dimer, trimer or even tetramer bands in non-reducing SDS-PAGE. Qβ capsid protein also shows unusual resistance to organic solvents and denaturing agents. It has been observed that DMSO and acetonitrile concentrations as high as 30%, and Guanidinium concentrations as high as 1 M do not affect the stability of the capsid. The high stability of the capsid of Qβ coat protein is an advantageous feature, in particular, for its use in immunization and vaccination of mammals and humans in accordance of the present invention.

Upon expression in *E. coli*, the N-terminal methionine of Qβ coat protein is usually removed, as observed by N-terminal Edman sequencing described in Stoll, E. et al. J. Biol. Chem.

252:990-993 (1977). VLP composed from Qβ coat proteins where the N-terminal methionine has not been removed, or VLPs comprising a mixture of Qβ coat proteins where the N-terminal methionine is either cleaved or present are also within the scope of the present disclosure.

Further RNA phage coat proteins have also been shown to self-assemble upon expression in a bacterial host (Kastelein, R A. et al., Gene 23: 245-254 (1983), Kozlovskaya, T M. et al., Dokl. Akad. Nauk SSSR 287: 452-455 (1986), Adhin, M R. et al., Virology 170: 238-242 (1989), Ni, C Z., et al., Protein Sci. 5: 2485-2493 (1996), Priano, C. et al., J. Mol. Biol. 249: 283-297 (1995)). The Qβ phage capsid contains, in addition to the coat protein, the so called read-through protein A1 and the maturation protein A2. A1 is generated by suppression at the UGA stop codon and has a length of 329 aa. In some embodiments, the capsid of phage Qβ recombinant coat protein used in the disclosure is devoid of the A2 lysis protein, and contains RNA from the host. The coat protein of RNA phages is an RNA binding protein, and interacts with the stem loop of the ribosomal binding site of the replicase gene acting as a translational repressor during the life cycle of the virus. The sequence and structural elements of the interaction are known (Witherell, G W. & Uhlenbeck, O C. Biochemistry 28: 71-76 (1989); Lim F. et al., J. Biol. Chem. 271: 31839-31845 (1996)). The stem loop and RNA in general are known to be involved in the virus assembly (Golmohammadi, R. et al., Structure 4: 543-5554 (1996)).

In some embodiments, the virus-like particle comprises recombinant proteins, or fragments thereof, of a RNA-phage, wherein the recombinant proteins comprise mutant coat proteins of a RNA phage, preferably of mutant coat proteins of the RNA phages mentioned above. In some embodiments, the mutant coat proteins of the RNA phage have been modified by removal of at least one lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution; alternatively, the mutant coat proteins of the RNA phage have been modified by deletion of at least one lysine residue, or by addition of at least one lysine residue by way of insertion.

In some embodiments, the virus-like particle comprises recombinant proteins, or fragments thereof, of the RNA-bacteriophage Qβ, wherein the recombinant proteins comprise coat proteins having an amino acid sequence of SEQ ID NO: 112, or a mixture of coat proteins having amino acid sequences of SEQ ID NO: 112 and of SEQ ID NO: 113 or mutants of SEQ ID NO: 113 and wherein the N-terminal methionine is preferably cleaved.

In some embodiments, the virus-like particle comprises recombinant proteins of Qβ or fragments thereof, wherein the recombinant proteins comprise mutant Qβ coat proteins. In some embodiments, these mutant coat proteins have been modified by removal of at least one lysine residue by way of substitution, or by addition of at least one lysine residue by way of substitution. Alternatively, these mutant coat proteins have been modified by deletion of at least one lysine residue, or by addition of at least one lysine residue by way of insertion.

Four lysine residues are exposed on the surface of the capsid of Qβ coat protein. Qβ mutants, for which exposed lysine residues are replaced by arginines can also be used for the present invention. The following Qβ coat protein mutants and mutant Qβ VLPs can, thus, be used in the practice of the invention: "Qβ 240" (Lys13-Arg; SEQ ID NO: 125), "Qβ-243" (Asn 10-Lys; SEQ ID NO: 126), "Qβ-250" (Lys 2-Arg, Lys13-Arg; SEQ ID NO: 127), "Qβ-251" (SEQ ID NO: 128) and "Qβ-259" (Lys 2-Arg, Lys16-Arg; SEQ ID NO: 129). Thus, in some embodiments, the virus-like particle comprises recombinant proteins of mutant Qβ coat proteins, which comprise proteins having an amino acid sequence selected from the group of a) the amino acid sequence of SEQ ID NO: 125; b) the amino acid sequence of SEQ ID NO: 126; c) the amino acid sequence of SEQ ID NO: 127; d) the amino acid sequence of SEQ ID NO: 128; and e) the amino acid sequence of SEQ ID NO: 129. The construction, expression and purification of the above indicated Qβ coat proteins, mutant Qβ coat protein VLPs and capsids, respectively, are disclosed in US Publication US 2003-0175290, herein incorporated by this reference in its entirety. In particular is hereby referred to Example 18 of above mentioned application.

In some embodiments, the virus-like particle comprises recombinant proteins of Qβ, or fragments thereof, wherein the recombinant proteins comprise a mixture of either one of the foregoing Qβ mutants and the corresponding A1 protein.

In some embodiments, the virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage AP205.

The AP205 genome consists of a maturation protein, a coat protein, a replicase and two open reading frames not present in related phages; a lysis gene and an open reading frame playing a role in the translation of the maturation gene (Klovins, J., et al., J. Gen. Virol. 83: 1523-33 (2002)). AP205 coat protein can be expressed from plasmid pAP283-58 (SEQ ID NO: 79), which is a derivative of pQb10 (Kozlovska, T. M. et al., Gene 137:133-37 (1993)), and which contains an AP205 ribosomal binding site. Alternatively, AP205 coat protein may be cloned into pQb185,

downstream of the ribosomal binding site present in the vector. Both approaches lead to expression of the protein and formation of capsids as described in US Patent No. 7,138,252, which is incorporated by reference in its entirety. Vectors pQb10 and pQb185 are vectors derived from pGEM vector, and expression of the cloned genes in these vectors is controlled by the trp promoter (Kozlovska, T. M. et al., Gene 137:133-37 (1993)). Plasmid pAP283-58 (SEQ ID NO: 130) comprises a putative AP205 ribosomal binding site in the following sequence, which is downstream of the XbaI site, and immediately upstream of the ATG start codon of the AP205 coat protein: tctagaATTTTCTGCGCACCCATCCCGGGTGGCGCCCAAAGTGAGGAAAATCAC atg (SEQ ID NO: 131). The vector pQb185 comprises a Shine Delagarno sequence downstream from the XbaI site and upstream of the start codon (tctagaTTAACCCAACGCGTAGGAGTCAGGCCatg, Shine Delagarno sequence underlined, SEQ ID NO: 132).

In some embodiments, the virus-like particle comprises recombinant coat proteins, or fragments thereof, of the RNA-phage AP205.

In some embodiments, AP205 coat proteins that form capsids. Such proteins are recombinantly expressed, or prepared from natural sources. AP205 coat proteins produced in bacteria spontaneously form capsids, as evidenced by Electron Microscopy (EM) and immunodiffusion. The structural properties of the capsid formed by the AP205 coat protein (SEQ ID NO: 133) and those formed by the coat protein of the AP205 RNA phage are nearly indistinguishable when seen in EM. AP205 VLPs are highly immunogenic, and can be linked with antigens and/or antigenic determinants to generate vaccine constructs displaying the antigens and/or antigenic determinants oriented in a repetitive manner. High titers are elicited against the so displayed antigens showing that bound antigens and/or antigenic determinants are accessible for interacting with antibody molecules and are immunogenic.

In some embodiments, the virus-like particle comprises recombinant mutant coat proteins, or fragments thereof, of the RNA-phage AP205.

In some embodiments, assembly-competent mutant forms of AP205 VLPs, including AP205 coat protein with the substitution of proline at amino acid 5 to threonine (SEQ ID NO: 134), are used in the practice of the disclosure. These VLPs, AP205 VLPs derived from natural sources, or AP205 viral particles, may be bound to antigens to produce ordered repetitive arrays of the antigens in accordance with the present invention.

AP205 P5-T mutant coat protein can be expressed from plasmid pAP281-32 (SEQ ID No. 135), which is derived directly from pQb185, and which contains the mutant AP205 coat protein gene instead of the Qβ coat protein gene. Vectors for expression of the AP205 coat protein are transfected into E. coli for expression of the AP205 coat protein.

In some embodiments, the disclosure provides compositions comprising proteins having amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to wild-type proteins which form ordered arrays and have an inherent repetitive structure.

In some embodiments, the disclosure provides nucleic acid molecules which encode proteins used to prepare compositions of the present invention.

In some embodiments, the compositions described herein comprise proteins comprising amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to any of the amino acid sequences shown in SEQ ID NOs:112-129.

Proteins suitable for use in the present disclosure also include C-terminal truncation mutants of proteins which form capsids or capsid-like structures, or VLPs. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs: 112-129 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the C-terminus. Typically, these C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Further proteins suitable for use in the present disclosure also include N-terminal truncation mutants of proteins which form capsids or capsid-like structures. Specific examples of such truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:112-129 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus. Typically, these N-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Additional proteins suitable for use in the present disclosure include N- and C-terminal truncation mutants which form capsids or capsid-like structures. Suitable truncation mutants include proteins having an amino acid sequence shown in any of SEQ ID NOs:112-129 where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus and 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the C-terminus. Typically, these N-terminal and C-terminal truncation mutants will retain the ability to form capsids or capsid-like structures.

Fragments of VLPs which retain the ability to induce an immune response can comprise, or alternatively consist of, polypeptides which are about 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450 or 500 amino acids in length, but will obviously depend on the length of the sequence of the subunit composing the VLP. Examples of such fragments include fragments of proteins discussed herein which are suitable for the preparation of the immune response enhancing composition.

In some embodiments, the VLP's are free of a lipoprotein envelope or a lipoprotein-containing envelope. In some embodiments, the VLP's are free of an envelope altogether.

The lack of a lipoprotein envelope or lipoprotein-containing envelope and, in particular, the complete lack of an envelope leads to a more defined virus-like particle in its structure and composition. Such more defined virus-like particles, therefore, may minimize side-effects. Moreover, the lack of a lipoprotein-containing envelope or, in particular, the complete lack of an envelope avoids or minimizes incorporation of potentially toxic molecules and pyrogens within the virus-like particle.

In some embodiments, the particles used in compositions of the disclosure are composed of a Hepatitis B capsid (core) protein (HBcAg) or a fragment of a HBcAg which has been modified to either eliminate or reduce the number of free cysteine residues. Zhou et al. (J. Virol. 66:5393-5398 (1992)) demonstrated that HBcAgs which have been modified to remove the naturally resident cysteine residues retain the ability to associate and form multimeric structures. Thus, core particles suitable for use in compositions of the disclosure include those comprising modified HBcAgs, or fragments thereof, in which one or more of the naturally resident cysteine residues have been either deleted or substituted with another amino acid residue (e.g., a serine residue).

The HBcAg is a protein generated by the processing of a Hepatitis B core antigen precursor protein. A number of isotypes of the HBcAg have been identified and their amino acids sequences are readily available to those skilled in the art. For example, the HBcAg protein having the amino acid sequence shown SEQ ID NO: 136 is 185 amino acids in length and is generated by the processing of a 212 amino acid Hepatitis B core antigen precursor protein. This processing results in the removal of 29 amino acids from the N-terminus of the Hepatitis B core antigen precursor protein. Similarly, the HBcAg protein that is 185 amino acids in length is generated by the processing of a 214 amino acid Hepatitis B core antigen precursor protein.

In some embodiments, compositions of the disclosure will be prepared using the processed form of a HBcAg (i.e., a HBcAg from which the N-terminal leader sequence of the Hepatitis B core antigen precursor protein have been removed).

Further, when HBcAgs are produced under conditions where processing will not occur, the HBcAgs will generally be expressed in "processed" form. For example, bacterial systems, such as E. coli, generally do not remove the leader sequences, also referred to as "signal peptides," of proteins which are normally expressed in eukaryotic cells. Thus, when an E. coli expression system directing expression of the protein to the cytoplasm is used to produce HBcAgs of the disclosure, these proteins will generally be expressed such that the N-terminal leader sequence of the Hepatitis B core antigen precursor protein is not present.

The preparation of Hepatitis B virus-like particles, which can be used for the present disclosure, is disclosed, for example, in WO 00/32227, and hereby in particular in Examples 17 to 19 and 21 to 24, as well as in WO 01/85208, and hereby in particular in Examples 17 to 19, 21 to 24, 31 and 41, and in pending U.S. Publication No. US 2003-0175290. For the latter application, it is in particular referred to Example 23, 24, 31 and 51. All three documents are explicitly incorporated herein by reference.

The present disclosure also includes HBcAg variants which have been modified to delete or substitute one or more additional cysteine residues. Thus, the vaccine compositions of the invention include compositions comprising HBcAgs in which cysteine residues not present in the amino acid sequence shown SEQ ID NO: 136 have been deleted.

It is well known in the art that free cysteine residues can be involved in a number of chemical side reactions. These side reactions include disulfide exchanges, reaction with chemical substances or metabolites that are, for example, injected or formed in a combination therapy with other substances, or direct oxidation and reaction with nucleotides upon exposure to UV light. Toxic adducts could thus be generated, especially considering the fact that HBcAgs have a strong tendency to bind nucleic acids. The toxic adducts would thus be distributed between a multiplicity of species, which individually may each be present at low concentration, but reach toxic levels when together.

In view of the above, one advantage to the use of HBcAgs in compositions which have been modified to remove naturally resident cysteine residues is that sites to which toxic species

can bind when antigens or antigenic determinants are attached would be reduced in number or eliminated altogether.

A number of naturally occurring HBcAg variants suitable for use in the practice of the present disclosure have been identified. Yuan et al., (J. Virol. 73:10122-10128 (1999)), for example, describe variants in which the isoleucine residue at position corresponding to position 97 in SEQ ID NO: 137 is replaced with either a leucine residue or a phenylalanine residue. The amino acid sequences of a number of HBcAg variants, as well as several Hepatitis B core antigen precursor variants, are disclosed in GenBank reports AAF121240 (SEQ ID NO: 138), AF121239 (SEQ ID NO: 139), X85297 (SEQ ID NO: 140), X02496 (SEQ ID NO: 141), X85305 (SEQ ID NO: 142), X85303 (SEQ ID NO: 143), AF151735 (SEQ ID NO: 144), X85259 (SEQ ID NO: 145), X85286 (SEQ ID NO: 146), X85260 (SEQ ID NO: 147), X85317 (SEQ ID NO: 148), X85298 (SEQ ID NO: 149), AF043593 (SEQ ID NO: 150), M20706 (SEQ ID NO: 151), X85295 (SEQ ID NO: 152), X80925 (SEQ ID NO: 153), X85284 (SEQ ID NO: 154), X85275 (SEQ ID NO: 155), X72702 (SEQ ID NO: 156), X85291 (SEQ ID NO: 157), X65258 (SEQ ID NO: 158), X85302 (SEQ ID NO: 159), M32138 (SEQ ID NO: 160), X85293 (SEQ ID NO: 161), X85315 (SEQ ID NO: 162), U95551 (SEQ ID NO: 163), X85256 (SEQ ID NO: 164), X85316 (SEQ ID NO: 165), X85296 (SEQ ID NO: 166), AB033559 (SEQ ID NO: 167), X59795 (SEQ ID NO: 168), X85299 (SEQ ID NO: 169), X85307 (SEQ ID NO: 170), X65257 (SEQ ID NO: 171), X85311 (SEQ ID NO: 172), X85301 (SEQ ID NO: 173), X85314 (SEQ ID NO: 174), X85287 (SEQ ID NO: 175), X85272 (SEQ ID NO: 176), X85319 (SEQ ID NO: 177), AB010289 (SEQ ID NO: 178), X85285 (SEQ ID NO: 179), AB010289 (SEQ ID NO:180), AF121242 (SEQ ID NO: 181), M90520 (SEQ ID NO: 182), P03153 (SEQ ID NO: 183), AF110999 (SEQ ID NO: 184), and M95589 (SEQ ID NO: 185), the disclosures of each of which are incorporated herein by reference. These HBcAg variants differ in amino acid sequence at a number of positions, including amino acid residues which corresponds to the amino acid residues located at positions 12, 13, 21, 22, 24, 29, 32, 33, 35, 38, 40, 42, 44, 45, 49, 51, 57, 58, 59, 64, 66, 67, 69, 74, 77, 80, 81, 87, 92, 93, 97, 98, 100, 103, 105, 106, 109, 113, 116, 121, 126, 130, 133, 135, 141, 147, 149, 157, 176, 178, 182 and 183 in SEQ ID NO:77. Further HBcAg variants suitable for use in the compositions of the invention, and which may be further modified according to the disclosure of this specification are described in WO 00/198333, WO 00/177158 and WO 00/214478.

HBcAgs suitable for use in the present disclosure can be derived from any organism so long as they are able to enclose or to be coupled or otherwise attached to, in particular as long as they are capable of packaging, a RLR agonist and induce an immune response.

In some embodiments, the compositions comprise an HBcAg variant capable of associating to form dimeric or multimeric structures. In some embodiments, the compositions comprise HBcAg polypeptides comprising amino acid sequences which are at least 80%, 85%, 90%, 95%, 97% or 99% identical to any of the wild-type amino acid sequences, and forms of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence.

Whether the amino acid sequence of a polypeptide has an amino acid sequence that is at least 80%, 85%, 90%, 95%, 97% or 99% identical to one of the wild-type amino acid sequences, or a subportion thereof, can be determined conventionally using known computer programs such the Bestfit program. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference amino acid sequence, the parameters are set such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The HBcAg variants and precursors having the amino acid sequences set out in SEQ ID NOs: 138-181 and 182-185 are relatively similar to each other. Thus, reference to an amino acid residue of a HBcAg variant located at a position which corresponds to a particular position in SEQ ID NO: 186, refers to the amino acid residue which is present at that position in the amino acid sequence shown in SEQ ID NO: 186. The homology between these HBcAg variants is for the most part high enough among Hepatitis B viruses that infect mammals so that one skilled in the art would have little difficulty reviewing the amino acid sequence shown in SEQ ID NO: 186 and in SEQ ID NO: 136, and that of a particular HBcAg variant and identifying "corresponding" amino acid residues. Furthermore, the HBcAg amino acid sequence shown in SEQ ID NO: 182, which shows the amino acid sequence of a HBcAg derived from a virus which infect woodchucks, has enough homology to the HBcAg having the amino acid sequence shown in SEQ ID NO: 186 that it is readily apparent that a three amino acid residue insert is present in SEQ ID NO: 182 between amino acid residues 155 and 156 of SEQ ID NO: 186.

As discussed above, the elimination of free cysteine residues reduces the number of sites where toxic components can bind to the HBcAg, and also eliminates sites where cross-linking of

lysine and cysteine residues of the same or of neighboring HBcAg molecules can occur. Therefore, in some embodiments, one or more cysteine residues of the Hepatitis B virus capsid protein have been either deleted or substituted with another amino acid residue.

In some embodiments, compositions described herein comprise HBcAgs from which the C-terminal region (e.g., amino acid residues 145-185 or 150-185 of SEQ ID NO: 186) has been removed. Thus, additional modified HBcAgs suitable for use in the practice of the present disclosure include C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 5, 10, 15, 20, 25, 30, 34, 35, amino acids have been removed from the C-terminus.

HBcAgs suitable for use in the practice of the present disclosure also include N-terminal truncation mutants. Suitable truncation mutants include modified HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, or 17 amino acids have been removed from the N-terminus.

Further HBcAgs suitable for use in the practice of the present disclosure include N- and C-terminal truncation mutants. Suitable truncation mutants include HBcAgs where 1, 2, 5, 7, 9, 10, 12, 14, 15, and 17 amino acids have been removed from the N-terminus and 1, 5, 10, 15, 20, 25, 30, 34, amino acids have been removed from the C-terminus.

In some embodiments, compositions comprising HBcAg polypeptides comprise amino acid sequences which are at least 80%, 85%, 90%, 95%, 97%, or 99% identical to the above described truncation mutants.

In some embodiments, a lysine residue is introduced into a HBcAg polypeptide, to mediate the binding of the antigen or antigenic determinant to the VLP of HBcAg. In some embodiments, compositions described herein are prepared using a HBcAg comprising amino acids 1-144, or 1-149, 1-185 of SEQ ID NO:186, which is modified so that the amino acids corresponding to positions 79 and 80 are replaced with a peptide having the amino acid sequence of Gly-Gly-Lys-Gly-Gly (SEQ ID NO:187). These compositions are particularly useful in those embodiments where an antigenic determinant is coupled to a VLP of HBcAg. In some embodiments, the cysteine residues at positions 48 and 107 of SEQ ID NO:186 are mutated to serine. In some embodiments, the compositions described herein comprise the corresponding polypeptides having amino acid sequences shown in any of SEQ ID NOs:138-183 which also have above noted amino acid alterations. Further included within the scope of the disclosure are additional HBcAg variants which are capable of associating to form a capsid or VLP and have the above noted amino acid alterations. Thus, the disclosure further includes compositions comprising HBcAg polypeptides

which comprise amino acid sequences at least 80%, 85%, 90%, 95%, 97% or 99% identical to any of the wild-type amino acid sequences, and forms of these proteins which have been processed, where appropriate, to remove the N-terminal leader sequence and modified with above noted alterations.

In some embodiments, compositions described herein comprise mixtures of different HBcAgs. Thus, these compositions may be composed of HBcAgs which differ in amino acid sequence. For example, compositions could be prepared comprising a "wild-type" HBcAg and a modified HBcAg in which one or more amino acid residues have been altered (e.g., deleted, inserted or substituted).

The crystal structure of several RNA bacteriophages has been determined (Golmohammadi, R. et al., Structure 4:543-554 (1996)). Using such information, surface exposed residues can be identified and, thus, RNA-phage coat proteins can be modified such that one or more reactive amino acid residues can be inserted by way of insertion or substitution. As a consequence, those modified forms of bacteriophage coat proteins can also be used for the present disclosure. Thus, variants of proteins which form capsids or capsid-like structures (e.g., coat proteins of bacteriophage Qβ, bacteriophage R17, bacteriophage fr, bacteriophage GA, bacteriophage SP, and bacteriophage MS2, bacteriophage AP 205) can also be used to prepare compositions described herein.

Although the sequence of the variants proteins discussed above will differ from their wild-type counterparts, these variant proteins will generally retain the ability to form capsids or capsid-like structures. Thus, the invention further includes compositions which further includes variants of proteins which form capsids or capsid-like structures, as well as methods for preparing such compositions, individual protein subunits used to prepare such compositions, and nucleic acid molecules which encode these protein subunits. Thus, included within the scope of the disclosure are variant forms of wild-type proteins which form capsids or capsid-like structures and retain the ability to associate and form capsids or capsid-like structures.

***Antigen and Antigenic Determinants***

In some embodiments, the compositions described herein comprise an antigen or antigenic determinant bound to the virus-like particle. The disclosure provides for compositions that vary according to the antigen or antigenic determinant selected in consideration of the desired

therapeutic effect. Exemplary antigens or antigenic determinants suitable for use in the present invention are disclosed in U.S. Patent No. 7,229,624, in U.S. Patent No. 6,964,769 and in U.S. Patent No. 7,264,810, the disclosures of which are herewith incorporated by reference in their entireties.

The antigen can be any antigen of known or yet unknown provenance. It can be isolated from bacteria, viruses or other pathogens or can be a recombinant antigen obtained from expression of suitable nucleic acid coding therefor. It can also be isolated from prions, tumors, self-molecules, non-peptidic hapten molecules, allergens and hormones. In some embodiments, the antigen is a recombinant antigen. The selection of the antigen is, of course, dependent upon the immunological response desired and the host.

In some embodiments, an immune response is induced against the VLP itself. In In some embodiments, a virus-like particle is coupled, fused or otherwise attached to an antigen/immunogen against which an enhanced immune response is desired.

In some embodiments, the at least one antigen or antigenic determinant is fused to the virus-like particle. As outlined above, a VLP is typically composed of at least one subunit assembling into a VLP. Thus, in some embodiments, the antigen or antigenic determinant is fused to at least one subunit of the virus-like particle or of a protein capable of being incorporated into a VLP generating a chimeric VLP-subunit-antigen fusion.

Fusion of the antigen or antigenic determinant can be effected by insertion into the VLP subunit sequence, or by fusion to either the N- or C-terminus of the VLP-subunit or protein capable of being incorporated into a VLP. Hereinafter, when referring to fusion proteins of a peptide to a VLP subunit, the fusion to either ends of the subunit sequence or internal insertion of the peptide within the subunit sequence are encompassed.

Fusion may also be effected by inserting antigen or antigenic determinant sequences into a variant of a VLP subunit where part of the subunit sequence has been deleted, that are further referred to as truncation mutants. Truncation mutants may have N- or C-terminal, or internal deletions of part of the sequence of the VLP subunit. For example, the specific VLP HBcAg with, for example, deletion of amino acid residues 79 to 81 is a truncation mutant with an internal deletion. In some embodiments, antigens or antigenic determinants are fused to either the N- or C-terminus of the truncation mutants VLP-subunits. Likewise, fusion of an epitope into the sequence of the VLP subunit may also be effected by substitution, where for example for the specific VLP

HBcAg, amino acids 79-81 are replaced with a foreign epitope. Thus, fusion, as referred to hereinafter, may be effected by insertion of the antigen or antigenic determinant sequence in the sequence of a VLP subunit, by substitution of part of the sequence of the VLP subunit with the antigen or antigenic determinant, or by a combination of deletion, substitution or insertions.

The chimeric antigen or antigenic determinant-VLP subunit will be in general capable of self-assembly into a VLP. VLP displaying epitopes fused to their subunits are also herein referred to as chimeric VLPs. As indicated, the virus-like particle comprises or alternatively is composed of at least one VLP subunit. In some embodiments, the virus-like particle comprises or alternatively is composed of a mixture of chimeric VLP subunits and non-chimeric VLP subunits, i.e. VLP subunits not having an antigen fused thereto, leading to so called mosaic particles. This may be advantageous to ensure formation of, and assembly to a VLP. In those embodiments, the proportion of chimeric VLP-subunits may be 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95% or higher.

Flanking amino acid residues may be added to either end of the sequence of the peptide or epitope to be fused to either end of the sequence of the subunit of a VLP, or for internal insertion of such peptidic sequence into the sequence of the subunit of a VLP. Glycine and serine residues are particularly favored amino acids to be used in the flanking sequences added to the peptide to be fused. Glycine residues confer additional flexibility, which may diminish the potentially destabilizing effect of fusing a foreign sequence into the sequence of a VLP subunit.

In some embodiments, the at least one antigen or antigenic determinant is fused to a Qβ coat protein. Fusion protein constructs wherein epitopes have been fused to the C-terminus of a truncated form of the A1 protein of Qβ or inserted within the A1 protein have been described (Kozlovska, T. M., et al., Intervirology, 39:9-15 (1996)). The A1 protein is generated by suppression at the UGA stop codon and has a length of 329 aa, or 328 aa, if the cleavage of the N-terminal methionine is taken into account. Cleavage of the N-terminal methionine before an alanine (the second amino acid encoded by the Qβ CP gene) usually takes place in E. coli, and such is the case for N-termini of the Qβ coat proteins. The part of the A1 gene, 3' of the UGA amber codon encodes the CP extension, which has a length of 195 amino acids. Insertion of the at least one antigen or antigenic determinant between position 72 and 73 of the CP extension leads to further embodiments of the invention (Kozlovska, T. M., et al., Intervirology 39:9-15 (1996)). Fusion of an antigen or antigenic determinant at the C-terminus of a C-terminally truncated Qβ A1

protein leads to further embodiments of the invention. For example, Kozlovska et al., (Intervirology, 39: 9-15 (1996)) describe Qβ A1 protein fusions where the epitope is fused at the C-terminus of the Qβ CP extension truncated at position 19.

As described by Kozlovska et al. (Intervirology, 39: 9-15 (1996)), assembly of the particles displaying the fused epitopes typically requires the presence of both the A1 protein-antigen fusion and the wild-type CP to form a mosaic particle. However, embodiments comprising virus-like particles, and hereby in particular the VLPs of the RNA phage Qβ coat protein, which are exclusively composed of VLP subunits having at least one antigen or antigenic determinant fused thereto, are also within the scope of the present disclosure.

The production of mosaic particles may be effected in a number of ways. Kozlovska et al., Intervirology, 39:9-15 (1996), describe three methods, which all can be used in the practice of the disclosure. In the first approach, efficient display of the fused epitope on the VLPs is mediated by the expression of the plasmid encoding the Qβ A1 protein fusion having a UGA stop codon between CP and CP extension in a E. coli strain harboring a plasmid encoding a cloned UGA suppressor tRNA which leads to translation of the UGA codon into Trp (pISM3001 plasmid (Smiley B. K., et al., Gene 134:33-40 (1993))). In another approach, the CP gene stop codon is modified into UAA, and a second plasmid expressing the A1 protein-antigen fusion is cotransformed. The second plasmid encodes a different antibiotic resistance and the origin of replication is compatible with the first plasmid (Kozlovska, T. M., et al., Intervirology 39:9-15 (1996)). In a third approach, CP and the A1 protein-antigen fusion are encoded in a bicistronic manner, operatively linked to a promoter such as the Trp promoter, as described in FIG. 1 of Kozlovska et al., Intervirology, 39:9-15 (1996).

In some embodiments, recombinant DNA technology can be utilized to fuse a heterologous protein to a VLP protein (Kratz, P. A., et al., Proc. Natl. Acad. Sci. USA 96:1915 (1999)). For example, the present disclosure encompasses VLPs recombinantly fused or chemically conjugated (including both covalently and non covalently conjugations) to an antigen (or portion thereof, preferably at least 10, 20 or 50 amino acids) to generate fusion proteins or conjugates. The fusion does not necessarily need to be direct, but can occur through linker sequences. More generally, in the case that epitopes, either fused, conjugated or otherwise attached to the virus-like particle, are used as antigens in accordance with the invention, spacer or linker sequences are typically added at one or both ends of the epitopes. Such linker sequences preferably comprise sequences

recognized by the proteasome, proteases of the endosomes or other vesicular compartment of the cell.

One way of coupling is by a peptide bond, in which the conjugate can be a contiguous polypeptide, i.e. a fusion protein. In some embodiments, different peptides or polypeptides are linked in frame to each other to form a contiguous polypeptide. Thus a first portion of the fusion protein comprises an antigen or immunogen and a second portion of the fusion protein, either N-terminal or C-terminal to the first portion, comprises a VLP. Alternatively, internal insertion into the VLP, with optional linking sequences on both ends of the antigen, can also be used in accordance with the present invention.

A flexible linker sequence (e.g. a polyglycine/polyserine-containing sequence such as [Gly4 Ser]2 (Huston et al., Meth. Enzymol 203:46-88 (1991)) can be inserted into the fusion protein between the antigen and ligand. Also, the fusion protein can be constructed to contain an "epitope tag", which allows the fusion protein to bind an antibody (e.g. monoclonal antibody) for example for labeling or purification purposes. An example of an epitope tag is a Glu-Glu-Phe tripeptide which is recognized by the monoclonal antibody YL1/2.

The disclosure also relates to the chimeric DNA which contains a sequence coding for the VLP and a sequence coding for the antigen/immunogen. The DNA can be expressed, for example, in insect cells transformed with Baculoviruses, in yeast or in bacteria. There are no restrictions regarding the expression system, of which a large selection is available for routine use. Preferably, a system is used which allows expression of the proteins in large amounts. In general, bacterial expression systems are used on account of their efficiency. One example of a bacterial expression system suitable for use within the scope of the present invention is the one described by Clarke et al., J. Gen. Virol. 71: 1109-1117 (1990); Borisova et al., J. Virol. 67: 3696-3701 (1993); and Studier et al., Methods Enzymol. 185:60-89 (1990). An example of a suitable yeast expression system is the one described by Emr, Methods Enzymol. 185:231-3 (1990); Baculovirus systems, which have previously been used for preparing capsid proteins, are also suitable. Constitutive or inducible expression systems can be used. By the choice and possible modification of available expression systems it is possible to control the form in which the proteins are obtained.

In some embodiments, the at least one antigen or antigenic determinant is bound to the virus-like particle by at least one covalent bond. In some embodiments, the least one antigen or antigenic determinant is bound to the virus-like particle by at least one covalent bond, said covalent

bond being a non-peptide bond leading to an antigen or antigenic determinant array and antigen or antigenic determinant-VLP conjugate, respectively. This antigen or antigenic determinant array and conjugate, respectively, has typically and preferably a repetitive and ordered structure since the at least one antigen or antigenic determinant is bound to the VLP in an oriented manner. In some embodiments, equal and more than 120, equal and more than 180, more than 270, and equal and more than 360 antigens are bound to the VLP. The formation of a repetitive and ordered antigen or antigenic determinant-VLP array and conjugate, respectively, is ensured by an oriented and directed as well as defined binding and attachment, respectively, of the at least one antigen or antigenic determinant to the VLP as will become apparent in the following. Furthermore, the typical inherent highly repetitive and organized structure of the VLPs advantageously contributes to the display of the antigen or antigenic determinant in a highly ordered and repetitive fashion leading to a highly organized and repetitive antigen or antigenic determinant-VLP array and conjugate, respectively.

VLPs or capsids of Qβ coat protein display a defined number of lysine residues on their surface, with a defined topology with three lysine residues pointing towards the interior of the capsid and interacting with the RNA, and four other lysine residues exposed to the exterior of the capsid. These defined properties favor the attachment of antigens to the exterior of the particle, rather than to the interior of the particle where the lysine residues interact with RNA. VLPs of other RNA phage coat proteins also have a defined number of lysine residues on their surface and a defined topology of these lysine residues.

In some embodiments, the first attachment site is a lysine residue and/or the second attachment comprises sulfhydryl group or a cysteine residue. In some embodiments, the first attachment site is a lysine residue and the second attachment is a cysteine residue.

In some embodiments, the antigen or antigenic determinant is bound via a cysteine residue, to lysine residues of the VLP of RNA phage coat protein, and in particular to the VLP of Q□ □coat protein.

The use of the VLPs as carriers allow the formation of robust antigen arrays and conjugates, respectively, with variable antigen density. In particular, the use of VLPs of RNA phages, and hereby in particular the use of the VLP of RNA phage Qβ coat protein allows to achieve very high epitope density. In particular, a density of more than 1.5 epitopes per subunit has been reached by coupling for example the human Aβ 1-6 peptide to the VLP of Qβ coat protein (WO 2004/016282).

The preparation of compositions of VLPs of RNA phage coat proteins with a high epitope density can be effected using the teaching of this application. In some embodiments, when an antigen or antigenic determinant is coupled to the VLP Qβ coat protein, an average number of antigen or antigenic determinant per subunit of 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4 2.5, 2.6, 2.7, 2.8, 2.9, or higher is used.

The second attachment site, as defined herein, may be either naturally or non-naturally present with the antigen or the antigenic determinant. In the case of the absence of a suitable natural occurring second attachment site on the antigen or antigenic determinant, such a, then non-natural second attachment has to be engineered to the antigen.

As described above, four lysine residues are exposed on the surface of the VLP of Qβ coat protein. Typically these residues are derivatized upon reaction with a cross-linker molecule. In the instance where not all of the exposed lysine residues can be coupled to an antigen, the lysine residues which have reacted with the cross-linker are left with a cross-linker molecule attached to the .quadrature.-amino group after the derivatization step. This leads to disappearance of one or several positive charges, which may be detrimental to the solubility and stability of the VLP. By replacing some of the lysine residues with arginines, as in the disclosed Qβ coat protein mutants described below, we prevent the excessive disappearance of positive charges since the arginine residues do not react with the cross-linker. Moreover, replacement of lysine residues by arginines may lead to more defined antigen arrays, as fewer sites are available for reaction to the antigen.

In some embodiments, exposed lysine residues are replaced by arginines in the following Qβ coat protein mutants and mutant Qβ VLPs disclosed herein: Qβ-240 (Lys13-Arg; SEQ ID NO: 125), Qβ-250 (Lys 2-Arg, Lys13-Arg; SEQ ID NO: 127) and Qβ-259 (Lys 2-Arg, Lys16-Arg; SEQ ID NO: 129).

In some embodiments, a Qβ mutant coat protein comprises one additional lysine residue, suitable for obtaining even higher density arrays of antigens. This mutant Qβ coat protein, Qβ-243 (Asn 10-Lys; SEQ ID NO: 126), was cloned, the protein expressed, and the capsid or VLP isolated and purified, showing that introduction of the additional lysine residue is compatible with self-assembly of the subunits to a capsid or VLP. Thus, antigen or antigenic determinant arrays and conjugates, respectively, may be prepared using VLP of Qβ coat protein mutants. A particularly favored method of attachment of antigens to VLPs, and in particular to VLPs of RNA phage coat proteins is the linking of a lysine residue present on the surface of the VLP of RNA phage coat

proteins with a cysteine residue added to the antigen. In order for a cysteine residue to be effective as second attachment site, a sulfhydryl group must be available for coupling. Thus, a cysteine residue has to be in its reduced state, that is, a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instant where the cysteine residue to function as second attachment site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with e.g. DTT, TCEP or .β-mercaptoethanol is required. The concentration of reductand, and the molar excess of reductand over antigen has to be adjusted for each antigen. A titration range, starting from concentrations as low as 10μM or lower, up to 10 to 20 mM or higher reductand if required is tested, and coupling of the antigen to the carrier assessed. Although low concentrations of reductand are compatible with the coupling reaction as described in WO 02/056905, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in which case the reductand has to be removed or its concentration decreased, e.g. by dialysis, gel filtration or reverse phase HPLC. Advantageously, the pH of the dialysis or equilibration buffer is lower than 7, preferably 6. The compatibility of the low pH buffer with antigen activity or stability has to be tested.

Epitope density on the VLP of RNA phage coat proteins can be modulated by the choice of cross-linker and other reaction conditions. For example, the cross-linkers Sulfo-GMBS and SMPH typically allow reaching high epitope density. Derivatization is positively influenced by high concentration of reactands, and manipulation of the reaction conditions can be used to control the number of antigens coupled to VLPs of RNA phage coat proteins, and in particular to VLPs of Qβ coat protein.

Prior to the design of a non-natural second attachment site the position at which it should be fused, inserted or generally engineered has to be chosen. The selection of the position of the second attachment site may, by way of example, be based on a crystal structure of the antigen. Such a crystal structure of the antigen may provide information on the availability of the C- or N-termini of the molecule (determined for example from their accessibility to solvent), or on the exposure to solvent of residues suitable for use as second attachment sites, such as cysteine residues. Exposed disulfide bridges, as is the case for Fab fragments, may also be a source of a second attachment site, since they can be generally converted to single cysteine residues through mild reduction, with e.g. 2-mercaptoethylamine, TCEP, β-mercaptoethanol or DTT. Mild reduction conditions not affecting the immunogenicity of the antigen will be chosen. In general,

in the case where immunization with a self-antigen is aiming at inhibiting the interaction of this self-antigen with its natural ligands, the second attachment site will be added such that it allows generation of antibodies against the site of interaction with the natural ligands. Thus, the location of the second attachment site will be selected such that steric hindrance from the second attachment site or any amino acid linker containing the same is avoided. In further embodiments, an antibody response directed at a site distinct from the interaction site of the self-antigen with its natural ligand is desired. In such embodiments, the second attachment site may be selected such that it prevents generation of antibodies against the interaction site of the self-antigen with its natural ligands.

Other criteria in selecting the position of the second attachment site include the oligomerization state of the antigen, the site of oligomerization, the presence of a cofactor, and the availability of experimental evidence disclosing sites in the antigen structure and sequence where modification of the antigen is compatible with the function of the self-antigen, or with the generation of antibodies recognizing the self-antigen.

In some embodiments, the antigen or antigenic determinant comprises a single second attachment site or a single reactive attachment site capable of association with the first attachment sites on the core particle and the VLPs or VLP subunits, respectively. This further ensures a defined and uniform binding and association, respectively, of the at least one, but typically more than one, preferably more than 10, 20, 40, 80, 120, 150, 180, 210, 240, 270, 300, 360, 400, 450 antigens to the core particle and VLP, respectively. The provision of a single second attachment site or a single reactive attachment site on the antigen, thus, ensures a single and uniform type of binding and association, respectively leading to a very highly ordered and repetitive array. For example, if the binding and association, respectively, is effected by way of a lysine- (as the first attachment site) and cysteine- (as a second attachment site) interaction, it is ensured, in accordance with one embodiment of the invention, that only one cysteine residue per antigen, independent whether this cysteine residue is naturally or non-naturally present on the antigen, is capable of binding and associating, respectively, with the VLP and the first attachment site of the core particle, respectively.

In some embodiments, engineering of a second attachment site onto the antigen require the fusion of an amino acid linker containing an amino acid suitable as second attachment site according to the disclosures of this invention. Therefore, in some embodiments, an amino acid linker is bound to the antigen or the antigenic determinant by way of at least one covalent bond.

In some embodiments, the amino acid linker comprises the second attachment site. In some embodiments, the amino acid linker comprises a sulfhydryl group or a cysteine residue. In some embodiments, the amino acid linker is cysteine.

In some embodiments, the virus-like particle comprises at least one first attachment site and the antigen or antigenic determinant comprises at least one second attachment site. In some embodiments, the first attachment site comprises an amino group or a lysine residue. In some embodiments, the second attachment site is selected from the group consisting of (a) an attachment site not naturally occurring with said antigen or antigenic determinant; and (b) an attachment site naturally occurring with said antigen or antigenic determinant. In some embodiments, the second attachment site comprises a sulfhydryl group or a cysteine residue. In some embodiments, the binding of the antigen or antigenic determinant to the virus-like particle is effected through association between the first attachment site and the second attachment site, wherein the association is through at least one non-peptide bond, and wherein the antigen or antigenic determinant and the virus-like particle interact through said association to form an ordered and repetitive antigen array. In some embodiments, the first attachment site is a lysine residue and the second attachment site is a cysteine residue. In some embodiments, the first attachment site is an amino group and the second attachment site is a sulfhydryl group.

The present disclosure is applicable to a wide variety of antigens. In some embodiments, the antigen is a protein, polypeptide or peptide. In some embodiments, the antigen is DNA. The antigen can also be a lipid, a carbohydrate, or an organic molecule, in particular a small organic molecule such as nicotine.

***Methods for Making VLPs and Packaging RLR Agonists in VLPs***

Methods for expression of the coat protein and the mutant coat protein, respectively, leading to self-assembly into VLPs are described in US Patent No. 7,138,252, which is incorporated by reference in its entirety. Suitable E. coli strains include, but are not limited to, E. coli K802, JM 109, RR1. Suitable vectors and strains and combinations thereof can be identified by testing expression of the coat protein and mutant coat protein, respectively, by SDS-PAGE and capsid formation and assembly by optionally first purifying the capsids by gel filtration and subsequently testing them in an immunodiffusion assay (Ouchterlony test) or Electron Microscopy (Kozlovska, T. M. et al., Gene 137:133-37 (1993)).

An advantage of using VLPs derived from RNA phages is their high expression yield in bacteria that allows production of large quantities of material at affordable cost. Methods for making the virus-like particles described herein, including methods scalable to a commercial scale, are described in US Patent Nos. 9,518,095 and 9,657,065, herein incorporated by reference in their entirety.

The disclosure also provides a method of producing a composition comprising a VLP and a RLR agonist package into the VLP which comprises incubating the VLP with the RLR agonist, adding RNase and purifying said composition. In some embodiments, the method further comprises the step of binding an antigen or antigenic determinant to said virus-like particle. In some embodiments, the antigen or antigenic determinant is bound to the virus-like particle before incubating the virus-like particle with the RLR agonist. In some embodiments, the antigen or antigenic determinant is bound to the virus-like particle after purifying the composition. In some embodiments, the method comprises incubating the VLP with RNase, adding the RLR agonist and purifying the composition. In some embodiments, the method further comprises the step of binding an antigen or antigenic determinant to said virus-like particle. In some embodiments, the antigen or antigenic determinant is bound to the virus-like particle before incubating the virus-like particle with the RNase. In some embodiments, the antigen or antigenic determinant is bound to the virus-like particle after purifying the composition. In some embodiments, the VLP is produced in a bacterial expression system. In another embodiment, the RNase is RNase A.

The disclosure further provides a method of producing a composition comprising a RLR agonist packaged into a VLP, which comprises disassembling the VLP, adding the RLR agonist, and reassembling the VLP. In some embodiments, the disassembled VLP is produced when manufacturing the VLP. In some embodiments, disassembled VLP comprises isolated dimers of a coat protein (e.g., Qβ dimers). In some embodiments, the isolated dimers assemble into the VLP around the RLR agonist to package the agonist into the VLP. The method can further comprise removing nucleic acids of the disassembled VLP and/or purifying the composition after reassembly. In some embodiments, the method further comprises the step of binding an antigen or antigenic determinant to the virus-like particle. In some embodiments, the antigen or antigenic determinant is bound to the virus-like particle before disassembling the virus-like particle. In some embodiments, the antigen or antigenic determinant is bound to the virus-like particle after reassembling the virus-like particle and preferably after purifying the composition.

The present disclosure provides methods of binding of antigen or antigenic determinant to VLPs. As indicated, in some embodiments, the at least one antigen or antigenic determinant is bound to the VLP by way of chemical cross-linking, typically and preferably by using a heterobifunctional cross-linker. Several hetero-bifunctional cross-linkers are known to the art. In some embodiments, the hetero-bifunctional cross-linker contains a functional group which can react with first attachment sites, i.e. with the side-chain amino group of lysine residues of the VLP or at least one VLP subunit, and a further functional group which can react with a second attachment site, i.e. a cysteine residue fused to the antigen or antigenic determinant and optionally also made available for reaction by reduction. The first step of the procedure, typically called the derivatization, is the reaction of the VLP with the cross-linker. The product of this reaction is an activated VLP, also called activated carrier. In the second step, unreacted cross-linker is removed using usual methods such as gel filtration or dialysis. In the third step, the antigen or antigenic determinant is reacted with the activated VLP, and this step is typically called the coupling step. Unreacted antigen or antigenic determinant may be optionally removed in a fourth step, for example by dialysis. Several hetero-bifunctional cross-linkers are known to the art. These include the cross-linkers SMPH (Pierce), Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available for example from the Pierce Chemical Company (Rockford, Ill., USA), and having one functional group reactive towards amino groups and one functional group reactive towards cysteine residues. The above mentioned cross-linkers all lead to formation of a thioether linkage. Another class of cross-linkers suitable is characterized by the introduction of a disulfide linkage between the antigen or antigenic determinant and the VLP upon coupling. In one embodiment, cross-linkers belonging to this class include for example SPDP and Sulfo-LC-SPDP (Pierce). The extent of derivatization of the VLP with cross-linker can be influenced by varying experimental conditions such as the concentration of each of the reaction partners, the excess of one reagent over the other, the pH, the temperature and the ionic strength. The degree of coupling, i.e. the amount of antigens or antigenic determinants per subunits of the VLP can be adjusted by varying the experimental conditions described above to match the requirements of the vaccine.

In some embodiments, a method of binding of antigens or antigenic determinants to the VLP comprises linking of a lysine residue on the surface of the VLP with a cysteine residue on the antigen or antigenic determinant. In some embodiments, fusion of an amino acid linker containing

a cysteine residue, as a second attachment site or as a part thereof, to the antigen or antigenic determinant for coupling to the VLP may be required.

In some embodiments, flexible amino acid linkers are used. Examples of the amino acid linker are selected from the group consisting of: (a) CGG; (b) N-terminal gamma 1-linker; (c) N-terminal gamma 3-linker; (d) Ig hinge regions; (e) N-terminal glycine linkers; (f) (G)kC(G)n with n=0-12 and k=0-5; (g) N-terminal glycine-serine linkers; (h) (G)kC(G)m(S)l(GGGGS)n with n=0-3, k=0-5, m=0-10, l=0-2 (SEQ ID NO: 188); (i) GGC; (k) GGC-NH2; (l) C-terminal gamma 1-linker; (m) C-terminal gamma 3-linker; (n) C-terminal glycine linkers; (o) (G)nC(G)k with n=0-12 and k=0-5; (p) C-terminal glycine-serine linkers; (q) (G)m(S)l(GGGGS)n(G)oC(G)k with n=0-3, k=0-5, m=0-10, l=0-2, and o=0-8 (SEQ ID NO: 189).

Further examples of amino acid linkers are the hinge region of Immunoglobulins, glycine serine linkers (GGGGS)n (SEQ ID NO: 190), and glycine linkers (G)n all further containing a cysteine residue as second attachment site and optionally further glycine residues. Typical examples of said amino acid linkers are N-terminal gamma 1: CGDKTHTSPP (SEQ ID NO: 191); C-terminal gamma 1: DKTHTSPPCG (SEQ ID NO: 192); N-terminal gamma 3: CGGPKPSTPPGSSGGAP (SEQ ID NO: 193); C-terminal gamma 3: PKPSTPPGSSGGAPGGCG (SEQ ID NO: 194); N-terminal glycine linker: GCGGGG (SEQ ID NO: 195); C-terminal glycine linker: GGGGCG (SEQ ID NO: 196); C-terminal glycine-lysine linker: GGKKGC (SEQ ID NO: 197); N-terminal glycine-lysine linker: CGKKGG (SEQ ID NO: 198).

In some embodiments, other amino acid linkers when a hydrophobic antigen or antigenic determinant is bound to a VLP, are CGKKGG (SEQ ID NO: 199), or CGDEGG (SEQ ID NO: 200) for N-terminal linkers, or GGKKGC (SEQ ID NO: 201) and GGEDGC (SEQ ID NO: 202), for the C-terminal linkers. For the C-terminal linkers, the terminal cysteine is optionally C-terminally amidated.

In some embodiments, GGCG (SEQ ID NO: 203), GGC or GGC-NH2 ("NH2" stands for amidation) linkers at the C-terminus of the peptide or CGG at its N-terminus are used as amino acid linkers. In general, glycine residues will be inserted between bulky amino acids and the cysteine to be used as second attachment site, to avoid potential steric hindrance of the bulkier amino acid in the coupling reaction. In some embodiments, the amino acid linker GGC-NH2 is fused to the C-terminus of the antigen or antigenic determinant.

The cysteine residue present on the antigen or antigenic determinant has to be in its reduced state to react with the hetero-bifunctional cross-linker on the activated VLP, that is a free cysteine or a cysteine residue with a free sulfhydryl group has to be available. In the instance where the cysteine residue to function as binding site is in an oxidized form, for example if it is forming a disulfide bridge, reduction of this disulfide bridge with e.g. DTT, TCEP or β-mercaptoethanol is required. Low concentrations of reducing agent are compatible with coupling as described in WO 02/05690, higher concentrations inhibit the coupling reaction, as a skilled artisan would know, in which case the reductand has to be removed or its concentration decreased prior to coupling, e.g. by dialysis, gel filtration or reverse phase BPLC.

Binding of the antigen or antigenic determinant to the VLP by using a hetero-bifunctional cross-linker according to the methods described above, allows coupling of the antigen or antigenic determinant to the VLP in an oriented fashion. Other methods of binding the antigen or antigenic determinant to the VLP include methods wherein the antigen or antigenic determinant is cross-linked to the VLP using the carbodiimide EDC, and NHS. In further methods, the antigen or antigenic determinant is attached to the VLP using a homo-bifunctional cross-linker such as glutaraldehyde, DSG, BM[PEO]4, BS3, (Pierce Chemical Company, Rockford, Ill., USA) or other known homo-bifunctional cross-linkers with functional groups reactive towards amine groups or carboxyl groups of the VLP.

Other methods of binding the VLP to an antigen or antigenic determinant include methods where the VLP is biotinylated, and the antigen or antigenic determinant expressed as a streptavidin-fusion protein, or methods wherein both the antigen or antigenic determinant and the VLP are biotinylated, for example as described in WO 00/23955. In this case, the antigen or antigenic determinant may be first bound to streptavidin or avidin by adjusting the ratio of antigen or antigenic determinant to streptavidin such that free binding sites are still available for binding of the VLP, which is added in the next step. Alternatively, all components may be mixed in a "one pot" reaction. Other ligand-receptor pairs, where a soluble form of the receptor and of the ligand is available, and are capable of being cross-linked to the VLP or the antigen or antigenic determinant, may be used as binding agents for binding antigen or antigenic determinant to the VLP. Alternatively, either the ligand or the receptor may be fused to the antigen or antigenic determinant, and so mediate binding to the VLP chemically bound or fused either to the receptor, or the ligand respectively. Fusion may also be effected by insertion or substitution.

**Pharmaceutical Compositions and Formulations**

In certain embodiments, the invention provides for a pharmaceutical composition comprising an RLR agonist with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant.

In certain embodiments, acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed. In certain embodiments, the formulation material(s) are for s.c. and/or I.V. administration. In certain embodiments, the pharmaceutical composition can contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolality, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. In certain embodiments, suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium hydrogen- sulfite); buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates or other organic acids); bulking agents (such as mannitol or glycine); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta- cyclodextrin); fillers; monosaccharides; disaccharides; and other carbohydrates (such as glucose, mannose or dextrins); proteins (such as serum albumin, gelatin or immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides, preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. (Remington's Pharmaceutical Sciences, 18th Edition, A. R. Gennaro, ed., Mack Publishing Company (1995). In certain embodiments, the formulation comprises PBS; 20 mM NaOAC, pH 5.2, 50 mM NaCl; and/or 10 mM NAOAC, pH 5.2, 9%

Sucrose. In certain embodiments, the optimal pharmaceutical composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format and desired dosage. See, for example, Remington's Pharmaceutical Sciences, supra. In certain embodiments, such compositions may influence the physical state, stability, rate of *in vivo* release and/or rate of *in vivo* clearance of the RLR agonist.

In certain embodiments, the primary vehicle or carrier in a pharmaceutical composition can be either aqueous or non-aqueous in nature. For example, in certain embodiments, a suitable vehicle or carrier can be water for injection, physiological saline solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. In certain embodiments, the saline comprises isotonic phosphate-buffered saline. In certain embodiments, neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. In certain embodiments, pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which can further include sorbitol or a suitable substitute therefore. In certain embodiments, a composition comprising an RLR agonist can be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (Remington's Pharmaceutical Sciences, supra) in the form of a lyophilized cake or an aqueous solution. Further, in certain embodiments, a composition comprising an RLR agonist can be formulated as a lyophilizate using appropriate excipients such as sucrose.

In certain embodiments, the pharmaceutical composition can be selected for parenteral delivery. In certain embodiments, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the ability of one skilled in the art.

In certain embodiments, the formulation components are present in concentrations that are acceptable to the site of administration. In certain embodiments, buffers are used to maintain the composition at physiological pH or at a slightly lower pH, typically within a pH range of from about 5 to about 8.

In certain embodiments, when parenteral administration is contemplated, a therapeutic composition can be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising an RLR agonist, in a pharmaceutically acceptable vehicle. In certain embodiments, a vehicle for parenteral injection is sterile distilled water in which an RLR agonist is formulated as

a sterile, isotonic solution, and properly preserved. In certain embodiments, the preparation can involve the formulation of the desired molecule with a delivery vehicle or agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid, polyglycolic acid or polyethylenimine (e.g. JetPEI®)), beads or liposomes, that can provide for the controlled or sustained release of the product which can then be delivered via a depot injection. In certain embodiments, hyaluronic acid can also be used, and can have the effect of promoting sustained duration in the circulation. In certain embodiments, implantable drug delivery devices can be used to introduce the desired molecule.

In certain embodiments, a pharmaceutical composition can be formulated for inhalation. In certain embodiments, an RLR agonist can be formulated as a dry powder for inhalation. In certain embodiments, an inhalation solution comprising an RLR agonist can be formulated with a propellant for aerosol delivery. In certain embodiments, solutions can be nebulized. Pulmonary administration is further described in PCT application No. PCT/US94/001875, which describes pulmonary delivery of chemically modified proteins.

In certain embodiments, it is contemplated that formulations can be administered orally. In certain embodiments, an RLR agonist that is administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. In certain embodiments, a capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. In certain embodiments, at least one additional agent can be included to facilitate absorption of an RLR agonist. In certain embodiments, diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

In certain embodiments, a pharmaceutical composition can involve an effective quantity of an RLR agonist or RIG-VLP in a mixture with non-toxic excipients which are suitable for the manufacture of tablets. In certain embodiments, by dissolving the tablets in sterile water, or another appropriate vehicle, solutions can be prepared in unit-dose form. In certain embodiments, suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions will be evident to those skilled in the art, including formulations involving an RLR agonist or RIG-VLP in sustained- or controlled-delivery formulations. In certain embodiments, techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See for example, PCT Application No. PCT/US93/00829 which describes the controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions. In certain embodiments, sustained-release preparations can include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polylactides (U.S. Pat. No. 3,773,919 and EP 058,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22:547-556 (1983)), poly (2-hydroxyethyl-methacrylate) (Langer et al., J. Biomed. Mater. Res., 15: 167-277 (1981) and Langer, Chem. Tech., 12:98- 105 (1982)), ethylene vinyl acetate (Langer et al., supra) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). In certain embodiments, sustained release compositions can also include liposomes, which can be prepared by any of several methods known in the art. See, e.g., Eppstein et al, Proc. Natl. Acad. Sci. USA, 82:3688-3692 (1985); EP 036,676; EP 088,046 and EP 143,949.

The pharmaceutical composition to be used for *in vivo* administration typically is sterile. In certain embodiments, this can be accomplished by filtration through sterile filtration membranes. In certain embodiments, where the composition is lyophilized, sterilization using this method can be conducted either prior to or following lyophilization and reconstitution. In certain embodiments, the composition for parenteral administration can be stored in lyophilized form or in a solution. In certain embodiments, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

In certain embodiments, once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or as a dehydrated or lyophilized powder. In certain embodiments, such formulations can be stored either in a ready-to-use form or in a form (e.g., lyophilized) that is reconstituted prior to administration.

In certain embodiments, kits are provided for producing a single-dose administration unit. In certain embodiments, the kit can contain both a first container having a dried protein and a

second container having an aqueous formulation. In certain embodiments, kits containing single and multi-chambered pre-filled syringes (e.g., liquid syringes and lyosyringes) are included.

In certain embodiments, the effective amount of a pharmaceutical composition comprising an RLR agonist or RIG-VLP to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment, according to certain embodiments, will thus vary depending, in part, upon the molecule delivered, the indication for which an RLR agonist or RIG-VLP is being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient. In certain embodiments, the clinician can titer the dosage and modify the route of administration to obtain the optimal therapeutic effect.

In certain embodiments, the frequency of dosing will take into account the pharmacokinetic parameters of an RLR agonist or RIG-VLP in the formulation used. In certain embodiments, a clinician will administer the composition until a dosage is reached that achieves the desired effect. In certain embodiments, the composition can therefore be administered as a single dose or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them. In certain embodiments, appropriate dosages can be ascertained through use of appropriate dose-response data.

In certain embodiments, the route of administration of the pharmaceutical composition is in accord with known methods, e.g. orally, through injection by intravenous, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, subcutaneously, intra-ocular, intraarterial, intraportal, or intralesional routes; by sustained release systems or by implantation devices. In certain embodiments, the compositions can be administered by bolus injection or continuously by infusion, or by implantation device. In certain embodiments, individual elements of the combination therapy may be administered by different routes.

In certain embodiments, the composition can be administered locally via implantation of a membrane, sponge or another appropriate material onto which the desired molecule has been absorbed or encapsulated. In certain embodiments, where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration. In certain embodiments, it can

be desirable to use a pharmaceutical composition comprising an RLR agonist in an *ex vivo* manner. In such instances, cells, tissues and/or organs that have been removed from the patient are exposed to a pharmaceutical composition comprising an RLR agonist or RIG-VLP after which the cells, tissues and/or organs are subsequently implanted back into the patient.

In certain embodiments, an RLR agonist or RIG-VLP can be delivered by implanting certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the agonist. In certain embodiments, such cells can be animal or human cells, and can be autologous, heterologous, or xenogeneic. In certain embodiments, the cells can be immortalized. In certain embodiments, in order to decrease the chance of an immunological response, the cells can be encapsulated to avoid infiltration of surrounding tissues. In certain embodiments, the encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow the release of the protein product(s) but prevent the destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

In some aspects, the disclosure provides a pharmaceutical composition comprising an RLR agonist or RIG-VLP according to the disclosure for stimulating an immune response, treating or delaying progression of a cancer, or reducing or inhibiting tumor growth in a subject in need thereof, and a pharmaceutically acceptable carrier. In some embodiments, the RLR agonist is formulated in a polyethylenimine (PEI) carrier. In some embodiments, the PEI carrier is JetPEI®.

**Applications**

The compositions described herein can be used in diagnostic and therapeutic applications. For example, detectably-labeled RLR agonists or RIG-VLPs can be used in assays to detect the presence or amount of the target protein in a sample (e.g., a biological sample). The compositions can be used in *in vitro* assays for studying inhibition of target function (e.g. RLR-mediated cellular signaling or response). In some embodiments, e.g., in which the compositions bind to and activate a target (e.g. a protein or polypeptide), the compositions can be used as positive controls in assays designed to identify additional novel compounds that also induce activity of the target protein or polypeptide and/or are otherwise are useful for treating a disorder associated with the target protein or polypeptide. For example, a RLR-activating composition can be used as a positive control in an assay to identify additional compounds (e.g., small molecules, aptamers, or antibodies) that

induce, increase, or stimulate RLR function. The compositions can also be used in therapeutic methods as elaborated on below.

**Kits**

A kit can include an RLR agonist or RIG-VLP as disclosed herein, and instructions for use. The kits may comprise, in a suitable container, an RLR agonist, one or more controls, and various buffers, reagents, enzymes and other standard ingredients well known in the art.

The container can include at least one vial, well, test tube, flask, bottle, syringe, or other container means, into which an RLR agonist or RIG-VLP may be placed, and in some instances, suitably aliquoted. Where an additional component is provided, the kit can contain additional containers into which this component may be placed. The kits can also include a means for containing an RLR agonist or RIG-VLP and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained. Containers and/or kits can include labeling with instructions for use and/or warnings.

In some aspects, the disclosure provides a kit comprising an RLR agonist or RIG-VLP provided by the disclosure, or comprising a pharmaceutical composition provided by the disclosure and instructions for use in stimulating an immune response in a subject, or treating or delaying progression of a cancer, or inhibiting tumor growth in a subject, optionally with instructions for use in combination with one or more additional therapeutic agents.

In some embodiments, the agonist or pharmaceutical composition is administered in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a cytokine, an activator of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof.

In some embodiments, the RLR agonist or pharmaceutical composition is administered preceding or subsequent to administration of the one or more additional therapeutic agents or wherein the one or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the RLR agonist or pharmaceutical composition.

In some embodiments, the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist, a TIM-3 antagonist, a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, a KIR antagonist, a LAG-3 antagonist, a toll-like receptor 3 (TLR3) agonist, a toll-like receptor 7 (TLR7) agonist, a toll-like receptor 9 (TLR9) agonist.

In some embodiments, the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g, antibody, or antigen binding portion thereof) that specifically binds to CD137 (4-1BB).

In some embodiments, the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD134 (OX40).

**Methods of Use**

The compositions of the present invention have numerous *in vitro* and *in vivo* utilities involving the detection and/or quantification of RLRs and/or the agonism of RLR function.

The above-described compositions are useful in, *inter alia*, methods for treating or preventing a variety of cancers or infectious diseases in a subject. The compositions can be administered to a subject, e.g., a human subject, using a variety of methods that depend, in part, on the route of administration. The route can be, e.g., intravenous injection or infusion (IV), subcutaneous injection (SC), intradermal injection (ID), intraperitoneal (IP) injection, intramuscular injection (IM), intratumoral injection (IT) or intrathecal injection. The injection can be in a bolus or a continuous infusion.

Administration can be achieved by, e.g., local infusion, injection, or by means of an implant. The implant can be of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. The implant can be configured for sustained or periodic release of the composition to the subject. See, e.g., U.S. Patent Application Publication No. 20080241223; U.S. Patent Nos. 5,501,856; 4,863,457; and 3,710,795; EP488401; and EP 430539, the disclosures of each of which are incorporated herein by reference in their entirety. The composition can be delivered to the subject by way of an implantable device based on, e.g., diffusive, erodible, or convective systems, e.g., osmotic pumps, biodegradable implants,

electrodiffusion systems, electroosmosis systems, vapor pressure pumps, electrolytic pumps, effervescent pumps, piezoelectric pumps, erosion-based systems, or electromechanical systems.

In some embodiments, an RLR agonist is therapeutically delivered to a subject by way of local administration. In some embodiments, an RLR agonist is packaged into a VLP. In one embodiment, the RLR agonist is therapeutically delivered with an antigen. In other embodiments, the VLP may be coupled to an antigen. In other embodiments, the RLR agonist is packaged into a VLP and administered in combination with a separate VLP coupled to an antigen.

A suitable dose of an RLR agonist or RIG-VLP described herein, which dose is capable of treating or preventing cancer in a subject, can depend on a variety of factors including, e.g., the age, sex, and weight of a subject to be treated and the particular inhibitor compound used. Other factors affecting the dose administered to the subject include, e.g., the type or severity of the cancer or infectious disease. For example, a subject having metastatic melanoma may require administration of a different dosage of an RLR agonist or RIG-VLP than a subject with glioblastoma. Other factors can include, e.g., other medical disorders concurrently or previously affecting the subject, the general health of the subject, the genetic disposition of the subject, diet, time of administration, rate of excretion, drug combination, and any other additional therapeutics that are administered to the subject. It should also be understood that a specific dosage and treatment regimen for any particular subject will also depend upon the judgment of the treating medical practitioner (e.g., doctor or nurse). Suitable dosages are described herein.

A pharmaceutical composition can include a therapeutically effective amount of an RLR agonist or RIG-VLP thereof described herein. Such effective amounts can be readily determined by one of ordinary skill in the art based, in part, on the effect of the administered RLR agonist or RIG-VLP, or the combinatorial effect of the RLR agonist or RIG-VLP and one or more additional active agents, if more than one agent is used. A therapeutically effective amount of an RLR agonist or RIG-VLP described herein can also vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the agonist (and one or more additional active agents) to elicit a desired response in the individual, e.g., reduction in tumor growth. For example, a therapeutically effective amount of an RLR agonist or RIG-VLP can inhibit (lessen the severity of or eliminate the occurrence of) and/or prevent a particular disorder, and/or any one of the symptoms of the particular disorder known in the art or described herein. A therapeutically

effective amount is also one in which any toxic or detrimental effects of the composition are outweighed by the therapeutically beneficial effects.

Suitable human doses of any of the RLR agonists or RIG-VLP described herein can further be evaluated in, e.g., Phase I dose escalation studies. See, e.g., van Gurp et al. (2008) *Am J Transplantation* 8(8):1711-1718; Hanouska et al. (2007) *Clin Cancer Res* 13(2, part 1):523-531; and Hetherington et al. (2006) *Antimicrobial Agents and Chemotherapy* 50(10): 3499-3500.

In some embodiments, the composition contains any of the RLR agonists or RIG-VLPs described herein and one or more (e.g., two, three, four, five, six, seven, eight, nine, 10, or 11 or more) additional therapeutic agents such that the composition as a whole is therapeutically effective. For example, a composition can contain an RLR agonist or RIG-VLP described herein and an alkylating agent, wherein the agonist and agent are each at a concentration that when combined are therapeutically effective for treating or preventing a cancer (e.g., melanoma) in a subject.

Toxicity and therapeutic efficacy of such compositions can be determined by known pharmaceutical procedures in cell cultures or experimental animals (e.g., animal models of any of the cancers described herein). These procedures can be used, e.g., for determining the $LD_{50}$ (the dose lethal to 50% of the population) and the $ED_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio $LD_{50}/ED_{50}$. An RLR agonist or RIG-VLP that exhibits a high therapeutic index is preferred. While compositions that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue and to minimize potential damage to normal cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. For an RLR agonist or RIG-VLP described herein, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the $EC_{50}$ (i.e., the concentration of the agonist which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography. In some embodiments, e.g., where local administration (e.g.,

to the eye or a joint) is desired, cell culture or animal modeling can be used to determine a dose required to achieve a therapeutically effective concentration within the local site.

In some embodiments, the methods can be performed in conjunction with other therapies for cancer or infectious disease. For example, the composition can be administered to a subject at the same time, prior to, or after, radiation, surgery, targeted or cytotoxic chemotherapy, chemoradiotherapy, hormone therapy, immunotherapy, gene therapy, cell transplant therapy, precision medicine, genome editing therapy, or other pharmacotherapy.

As described above, the compositions described herein (e.g., RLR agonist or RIG-VLP compositions) can be used to treat a variety of cancers such as but not limited to: Kaposi's sarcoma, leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, myeloblasts promyelocyte myelomonocytic monocytic erythroleukemia, chronic leukemia, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, mantle cell lymphoma, primary central nervous system lymphoma, Burkitt’s lymphoma, marginal zone B cell lymphoma, polycythemia vera, Hodgkin's disease, non-Hodgkin' s disease, multiple myeloma, Waldenstrom's macroglobulinemia, heavy chain disease, solid tumors, sarcomas, and carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chrondrosarcoma, osteogenic sarcoma, osteosarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon sarcoma, colorectal carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, retinoblastoma, nasopharyngeal carcinoma, esophageal carcinoma, basal cell carcinoma, biliary tract cancer, bladder cancer, bone cancer, brain and central nervous system (CNS) cancer, cervical cancer, choriocarcinoma, colorectal cancers, connective tissue cancer, cancer of the digestive system, endometrial cancer, esophageal cancer, eye cancer, head and neck cancer, gastric cancer, intraepithelial neoplasm,

kidney cancer, larynx cancer, liver cancer, lung cancer (small cell, large cell), melanoma, neuroblastoma; oral cavity cancer (for example lip, tongue, mouth and pharynx), ovarian cancer, pancreatic cancer, rectal cancer; cancer of the respiratory system, sarcoma, skin cancer, stomach cancer, testicular cancer, thyroid cancer, uterine cancer, and cancer of the urinary system.

In some embodiments, the disclosure provides a vaccine comprising an RLR agonist packaged into a VLP, and an antigen or antigenic determinant bound to the VLP. In some embodiments, the vaccine induces an immune response against the antigen or antigenic determinant bound to the VLP. In some embodiments, the vaccine is prophylactic. In some embodiments, the vaccine is therapeutic. In some embodiments, the antigen or antigenic determinant bound to the VLP is a cancer or tumor antigen, and thus the vaccine induces an anti-tumor immune response. In some embodiments, the vaccine induces protective immunity.

In some aspects, the disclosure provides a method to increase RLR-mediated production of one or more cytokines in a cell, the method comprising contacting the cell with an RLR agonist or RIG-VLP provided by the disclosure, wherein the agonist increases RLR-mediated cytokine production in a cell.

In some aspects, the disclosure provides a method to increase RLR-mediated expression of one or more interferon-stimulated genes in a cell, the method comprising contacting the cell with an RLR agonist or RIG-VLP provided by the disclosure, wherein the agonist increases RLR-mediated expression of one or more interferon-stimulated genes in a cell.

In some aspects, the disclosure provides a method to increase RLR-dependent intracellular signaling in a cell, the method comprising contacting the cell with an RLR agonist or RIG-VLP provided by the disclosure, wherein the agonist increases RLR-dependent intracellular signaling.

In some aspects, the disclosure provides a method of stimulating an immune response in a subject, the method comprising administering to the subject an effective amount of an RLR agonist or RIG-VLP provided by the disclosure, or a pharmaceutical composition provided by the disclosure.

In some aspects, the disclosure provides a method of treating or delaying progression of a cancer in a subject, the method comprising administering to the subject an effective amount of an RLR agonist or RIG-VLP provided by the disclosure, or a pharmaceutical composition provided by the disclosure.

In some aspects, the disclosure provides a method of reducing or inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject an effective amount of an RLR agonist or RIG-VLP provided by the disclosure, or a pharmaceutical composition provided by the disclosure.

In some aspects, the disclosure provides a method for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject an effective amount of an RLR agonist or RIG-VLP provided by the disclosure, or a pharmaceutical composition provided by the disclosure, wherein the agonist, or the pharmaceutical composition increases RLR-mediated production of one or more cytokines in a cell, increases RLR-mediated expression of one or more interferon-stimulated genes in a cell, and or increases RLR-dependent intracellular signaling in a cell, thereby stimulating the immune response, treating or delaying progression of the cancer, or inhibiting growth of the tumor.

**Combinations of RLR Agonists with Additional Therapeutic Agents**

In some embodiments, an RLR agonist or RIG-VLP described herein can be administered to a subject as a monotherapy. Alternatively, the RLR agonist or RIG-VLP can be administered to a subject as a combination therapy with another treatment, e.g., another treatment for a cancer. For example, the combination therapy can include administering to the subject (e.g., a human patient) one or more additional agents that provide a therapeutic benefit to a subject who has, or is at risk of developing, cancer.

In some embodiments of the methods provided by the disclosure, the RLR agonist or RIG-VLP or pharmaceutical composition is administered in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a cytokine, an activator or agonist of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof.

In some embodiments, combinations can be administered either concomitantly, e.g., as an admixture, separately but simultaneously or concurrently; or sequentially. This includes presentations in which the combined agents are administered together as a therapeutic mixture,

and also procedures in which the combined agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the compounds or agents given first, followed by the second.

In some embodiments, the RLR agonist, RIG-VLP, or pharmaceutical composition is administered preceding or subsequent to administration of the one or more additional therapeutic agents or wherein the one or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the agonist or pharmaceutical composition.

In some embodiments, the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist, a TIM-3 antagonist, a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, a KIR antagonist, a LAG-3 antagonist, a toll-like receptor 3 (TLR3) agonist, a toll-like receptor 7 (TLR7) agonist, a toll-like receptor 9 (TLR9) agonist.

***Combination with Chemotherapeutic Agents***

Chemotherapeutic agents suitable for combination and/or co-administration with compositions of the present invention include, for example: taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxyanthrancindione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Further agents include, for example, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g. mechlorethamine, thioTEPA, chlorambucil, melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, cis-dichlordiamine platinum (II)(DDP), procarbazine, altretamine, cisplatin, carboplatin, oxaliplatin, nedaplatin, satraplatin, or triplatin tetranitrate), anthracycline (e.g. daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g. dactinomcin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g. vincristine and vinblastine) and temozolomide.

***Combination with PD-1/PD-L1 Antagonists***

In some embodiments, a RLR agonist, RIG-VLP, or pharmaceutical compositions thereof, provided by the disclosure is combined (e.g., administered in combination) with one or more PD-1/PD-L1 antagonist that specifically binds to human PD-1 or PD-L1 and inhibits PD-1/PD-L1 biological activity and/or downstream pathway(s) and/or cellular processed mediated by human PD-1/PD-L1 signaling or other human PD-1/PD-L1-mediated functions.

Accordingly, provided herein are PD-1/PD-L1 antagonists that directly or allosterically block, antagonize, suppress, inhibit or reduce PD-1/PD-L1 biological activity, including downstream pathways and/or cellular processes mediated by PD-1/PD-L1 signaling, such as receptor binding and/or elicitation of a cellular response to PD-1/PD-L1. Also provided herein are PD-1/PD-L1 antagonists that reduce the quantity or amount of human PD-1 or PD-L1 produced by a cell or subject.

In some embodiments, the disclosure provides a PD-1/PD-L1 antagonist that binds human PD-1 and prevents, inhibits or reduces PD-L1 binding to PD-1. In some aspects, the PD-1/PD-L1 antagonist binds to the mRNA encoding PD-1 or PD-L1 and prevents translation. In some embodiments, the PD-1/PD-L1 antagonist binds to the mRNA encoding PD-1 or PD-L1 and causes degradation and/or turnover.

In some embodiments, the PD-1/PD-L1 antagonist inhibits PD-1 signaling or function. In some embodiments, the PD-1/PD-L1 antagonist blocks binding of PD-1 to PD-L1, PD-L2, or to both PD-L1 and PD-L2. In some embodiments, the PD-1/PD-L1 antagonist blocks binding of PD-1 to PD-L1. In some embodiments, the PD-1/PD-L1 antagonist blocks binding of PD-1 to PD-L2. In some embodiments, the PD-1/PD-L1 antagonist blocks the binding of PD-1 to PD-L1 and PD-L2. In some embodiments, the PD-1/PD-L1 antagonist specifically binds PD-1. In some embodiments, the PD-1/PD-L1 antagonist specifically binds PD-L1. In some embodiments, the PD-1/PD-L1 antagonist specifically binds PD-L2.

In some embodiments, the PD-1/PD-L1 antagonist inhibits the binding of PD-1 to its cognate ligand. In some embodiments, the PD-1/PD-L1 antagonist inhibits the binding of PD-1 to PD-L1, PD-1 to PD-L2, or PD-1 to both PD-L1 and PD-L2. In some embodiments, the PD-1/PD-L1 antagonist does not inhibit the binding of PD-1 to its cognate ligand.

In some embodiments, the PD-1/PD-L1 antagonist is an isolated monoclonal antibody (mAb), or antigen binding fragment thereof, which specifically binds to PD-1 or PD-L1. In some embodiments, the PD-1/PD-L1 antagonist is an antibody or antigen binding fragment thereof that

specifically binds to human PD-1. In some embodiments, the PD-1/PD-L1 antagonist is an antibody or antigen binding fragment thereof that specifically binds to human PD-L1. In some embodiments, the PD-1/PD-L1 antagonist is an antibody or antigen binding fragment that binds to human PD-L1 and inhibits the binding of PD-L1 to PD-1. In some embodiments, the PD-1/PD-L1 antagonist is an antibody or antigen binding fragment that binds to human PD-1 and inhibits the binding of PD-L1 to PD-1.

Several immune checkpoint antagonists that inhibit or disrupt the interaction between PD-1 and either one or both of its ligands PD-L1 and PD-L2 are in clinical development or are currently available to clinicians for treating cancer.

Examples of anti-human PD-1 monoclonal antibodies, or antigen binding fragments thereof, that may comprise the PD-1/PD-L1 antagonist in any of the compositions, methods, and uses provided by the disclosure include, but are not limited to: KEYTRUDA® (pembrolizumab, MK-3475, h409A11; see US8952136, US8354509, US8900587, and EP2170959, all of which are included herein by reference in their entirety; Merck), OPDIVO® (nivolumab, BMS-936558, MDX-1106, ONO-4538; see US7595048, US8728474, US9073994, US9067999, EP1537878, US8008449, US8779105, and EP2161336, all of which are included herein by reference in their entirety; Bristol Myers Squibb), MEDI0680 (AMP-514), BGB-A317 and BGB-108 (BeiGene), 244C8 and 388D4 (see WO2016106159, which is incorporated herein by reference in its entirety; Enumeral Biomedical), PDR001 (Novartis), and REGN2810 (Regeneron). Accordingly, in some embodiments the PD-1/PD-L1 antagonist is pembrolizumab. In some embodiments, the PD-1/PD-L1 antagonist is nivolumab.

Examples of anti-human PD-L1 monoclonal antibodies, or antigen binding fragments thereof, that may comprise the PD-1/PD-L1 antagonist in any of the compositions, methods, and uses provided by the disclosure include, but are not limited to: BAVENCIO® (avelumab, MSB0010718C, see WO2013/79174, which is incorporated herein by reference in its entirety; Merck/Pfizer), IMFINZI® (durvalumab, MEDI4736), TECENTRIQ® (atezolizumab, MPDL3280A, RG7446; see WO2010/077634, which is incorporated herein by reference in its entirety; Roche), MDX-1105 (BMS-936559, 12A4; see US7943743 and WO2013/173223, both of which are incorporated herein by reference in their entirety; Medarex/BMS), and FAZ053 (Novartis). Accordingly, in some embodiments the PD-1/PD-L1 antagonist is avelumab. In some

embodiments, the PD-1/PD-L1 antagonist is durvalumab. In some embodiments, the PD-1/PD-L1 antagonist is atezolizumab.

In some embodiments, the PD-1/PD-L1 antagonist is an immunoadhesin that specifically bind to human PD-1 or human PD-L1, e.g., a fusion protein containing the extracellular or PD-1 binding portion of PD-L1 or PD-L2 fused to a constant region such as an Fc region of an immunoglobulin molecule. Examples of immunoadhesion molecules that specifically bind to PD-1 are described in WO2010/027827 and WO2011/066342, both of which are incorporated herein by reference in their entirety. In some embodiments, the PD-1/PD-L1 antagonist is AMP-224 (also known as B7-DCIg), which is a PD-L2-FC fusion protein that specifically binds to human PD-1.

It will be understood by one of ordinary skill that any PD-1/PD-L1 antagonist which binds to PD-1 or PD-L1 and disrupts the PD-1/PD-L1 signaling pathway, is suitable for compositions, methods, and uses disclosed herein.

In some embodiments, the PD-1/PD-L1 antagonist is a small molecule, a nucleic acid, a peptide, a peptide mimetic, a protein, a carbohydrate, a carbohydrate derivative, or a glycopolymer. Exemplary small molecule PD-1 inhibitors are described in Zhan et al., (2016) Drug Discov Today 21(6):1027-1036.

In some embodiments of the methods provided by the disclosure, the RLR agonist is combined with a PD-1/PD-L1 antagonist, wherein the PD-1/PD-L1 antagonist is selected from the group consisting of: PDR001, KEYTRUDA® (pembrolizumab), OPDIVO® (nivolumab), pidilizumab, MEDI0680, REGN2810, TSR-042, PF-06801591, and AMP-224. In some embodiments, the PD-1/PD-L1 antagonist is selected from the group consisting of: FAZ053, TENCENTRIQ® (atezolizumab), BAVENCIO® (avelumab), IMFINZI® (durvalumab), and BMS-936559.

***Combinations with TIM-3 Antagonist***

In some embodiments, an RLR agonist, RIG-VLP, or pharmaceutical compositions thereof, provided by the disclosure is combined (e.g., administered in combination) with a TIM-3 antagonist. The TIM-3 antagonist may be an antibody, an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or an oligopeptide. In some embodiments, the TIM-3 antagonist is chosen from MGB453 (Novartis), TSR-022 (Tesaro), or LY3321367 (Eli Lilly).

***Combinations with LAG-3 Antagonist***

In some embodiments, an RLR agonist, RIG-VLP, or pharmaceutical compositions thereof, provided by the disclosure is combined (e.g., administered in combination) with a LAG-3 antagonist. The LAG-3 antagonist may be an antibody, an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide. In some embodiments, the LAG-3 inhibitor is chosen from LAG525 (Novartis), BMS-986016 (Bristol-Myers Squibb), TSR-033 (Tesaro), MK-4280 (Merck & Co), or REGN3767 (Regeneron).

***Combinations with Toll-Like Receptor (TLR) Agonists***

In some embodiments, an RLR agonist, RIG-VLP, or pharmaceutical composition thereof, provided by the disclosure is combined (e.g. administered in combination) with a TLR agonist.

Toll-like receptors (TLRs) are a family of germline-encoded transmembrane proteins that facilitate pathogen recognition and activation of the innate immune system. (Hoffmann et al., (1999) Science 284:1313-1318; Rock et al., (1998) Proc Natl Acad Sci USA 95:588-593). TLRs are pattern recognition receptors (PRRs), and are expressed by cells of the innate immune system. Examples of known ligands for TLRs include gram positive bacteria (TLR-2), bacterial endotoxin (TLR-4), flagellin protein (TLR-5), bacterial DNA (TLR-9), double-stranded RNA and poly I:C (TLR-3), and yeast (TLR-2). *In vivo* activation of TLRs initiates an innate immune response involving specific cytokines, chemokines and growth factors. While all TLRs can activate certain intracellular signaling molecules such as nuclear factor kappa beta (NF-κB) and mitogen activated protein kinases (MAP kinases), the specific set of cytokines and chemokines released appears to be unique for each TLR. TLR7, 8, and 9 comprise a subfamily of TLRs which are located in endosomal or lysosomal compartments of immune cells such as dendritic cells and monocytes. In contrast to TLR7 and 9 which are highly expressed on plasmacytoid dendritic cells (pDC), TLR8 is mainly expressed on myeloid DC (mDC) and monocytes. This subfamily mediates recognition of microbial nucleic acids, such as single stranded RNA.

Small, low-molecular weight (less than 400 Daltons) synthetic imidazoquinoline compounds which resemble the purine nucleotides adenosine and guanosine were the first TLR7 and TLR8 agonists to be identified. A number of these compounds have demonstrated anti-viral and anti-cancer properties. For example, the TLR7 agonist imiquimod (ALDARA™) was

approved by the U.S. Food and Drug Administration as a topical agent for the treatment of skin lesions caused by certain strains of the human papillomavirus. Imiquimod may also be useful for the treatment of primary skin cancers and cutaneous tumors such as basal cell carcinomas, keratoacanthomas, actinic keratoses, and Bowen's disease. The TLR7/8 agonist resiquimod (R-848) is being evaluated as a topical agent for the treatment of human genital herpes.

TLR agonists according to the disclosure can be any TLR agonist. For example, a TLR agonist can encompass a natural or synthetic TLR ligand, a mutein or derivative of a TLR ligand, a peptide mimetic of a TLR ligand, a small molecule that mimics the biological function of a TLR ligand, or an antibody that stimulates a TLR receptor. A TLR ligand is any molecule that binds to a TLR.

In some embodiments, an RLR agonist, RIG-VLP, or pharmaceutical composition thereof, provided by the disclosure, is combined with a TLR agonist, wherein the TLR agonist is selected from the group consisting of: a TLR1 agonist, a TLR2 agonist, a TLR3 agonist, a TLR4 agonist, a TLR5 agonist, a TLR6 agonist, a TLR7 agonist, a TLR8 agonist, a TLR9 agonist, a TLR10 agonist, and a TLR11 agonist.

In some embodiments, an RLR agonist or RIG-VLP provided by the disclosure is combined with a TLR3 agonist. A TLR3 agonist is an agonist that causes a signaling response through TLR3. Exemplary TLR3 agonists include, but are not limited to, polyinosinic:polycytidylic acid (poly I:C), HILTONOL® (poly ICLC), polyadenylic-polyuridylic acid (poly A:U), RIBOXXIM® (RGIC®100), RIBOXXON® (RGIC®50 bioconjugate), and RIBOXXOL® (RGIC®50).

In some embodiments, an RLR agonist or RIG-VLP provided by the disclosure is combined with polyinosinic:polycytidylic acid (poly I:C). In some embodiments, the RLR agonist or RIG-VLP is combined with HILTONOL® (poly ICLC). In some embodiments, the RLR agonist is combined with polyadenylic-polyuridylic acid (poly A:U). In some embodiments, the RLR agonist or RIG-VLP is combined with RIBOXXIM® (RGIC®100). In some embodiments, the RLR agonist or RIG-VLP is combined with RIBOXXON® (RGIC®50 bioconjugate). In some embodiments, the RLR agonist is combined with RIBOXXOL® (RGIC®50).

In some embodiments, an RLR agonist or RIG-VLP provided by the disclosure is combined with a TLR7 agonist. A TLR7 agonist is an agonist that causes a signaling response through TLR7. Non-limiting examples of TLR7 agonists include single stranded RNA (ssRNA),

loxoribine (a guanosine analogue derivatized at positions N7 and C8), imidazoquinoline compounds (e.g., imiquimod and resiquimod), or derivatives thereof. Further exemplary TLR7 agonists include, but are not limited to, GS-9620 (Vesatolimod), imiquimod (ALDARA™), and resiquimod (R-848).

In some embodiments, an RLR agonist or RIG-VLP provided by the disclosure is combined with GS-9620 (Vesatolimod). In some embodiments, the RLR agonist is combined with imiquimod (ALDARA™). In some embodiments, the RLR agonist or RIG-VLP is combined with resiquimod (R-848).

In some embodiments, an RLR agonist or RIG-VLP provided by the disclosure is combined with a TLR9 agonist. A TLR9 agonist is an agonist that causes a signaling response through TLR9. Exemplary TLR9 agonists include, but are not limited to, CpG oligodeoxynucleotides (GpG ODNs). In some embodiments, the CpG ODN is a Class A CpG ODN (CpG-A ODN), a Class B CpG ODN (CpG-B ODN), or a Class C CpG ODN (CpG-B ODN).

In some embodiments, an RLR agonist or RIG-VLP provided by the disclosure is combined with a CpG oligodeoxynucleotide (CpG ODN). In some embodiments, the CpG ODN is a Class A CpG ODN (CpG-A ODN). In some embodiments, the CpG ODN is a Class B CpG ODN (CpG-B ODN). In some embodiments, the CpG ODN is a Class C CpG ODN (CpG-C ODN).

***Other Combinations***

In some embodiments, an RLR agonist, RIG-VLP or pharmaceutical compositions thereof, provided by the disclosure is combined (e.g., administered in combination) with a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, or a KIR antagonist

In some embodiments, an RLR agonist, RIG-VLP or pharmaceutical compositions thereof, provided by the disclosure is combined (e.g., administered in combination) with an agonist comprising an polypeptide (e.g, antibody, or antigen binding portion thereof) that specifically binds to CD137 (4-1BB).

In some embodiments, an RLR agonist, RIG-VLP, or pharmaceutical compositions thereof, provided by the disclosure is combined (e.g., administered in combination) with an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD134 (OX40).

An RLR agonist or RIG-VLP described herein can replace or augment a previously or currently administered therapy. For example, upon treating with an RLR agonist or RIG-VLP, administration of the one or more additional active agents can cease or diminish, e.g., be administered at lower levels or dosages. In some embodiments, administration of the previous therapy can be maintained. In some embodiments, a previous therapy will be maintained until the level of the RLR agonist or RIG-VLP reaches a level sufficient to provide a therapeutic effect. The two therapies can be administered in combination.

Monitoring a subject (e.g., a human patient) for an improvement in a cancer, as defined herein, means evaluating the subject for a change in a disease parameter, e.g., a reduction in tumor growth. In some embodiments, the evaluation is performed at least one (1) hour, e.g., at least 2, 4, 6, 8, 12, 24, or 48 hours, or at least 1 day, 2 days, 4 days, 10 days, 13 days, 20 days or more, or at least 1 week, 2 weeks, 4 weeks, 10 weeks, 13 weeks, 20 weeks or more, after an administration. The subject can be evaluated in one or more of the following periods: prior to beginning of treatment; during the treatment; or after one or more elements of the treatment have been administered. Evaluation can include evaluating the need for further treatment, e.g., evaluating whether a dosage, frequency of administration, or duration of treatment should be altered. It can also include evaluating the need to add or drop a selected therapeutic modality, e.g., adding or dropping any of the treatments for a cancer described herein.

In some embodiments, an RLR agonist or RIG-VLP described herein is administered to modulate a T-cell response in a patient, for example, by increasing T-cell activation and/or proliferation. Enhancement of T cell proliferation, IFN production and secretion, and/or the cytolytic activity of T cells may be beneficial to patients in need thereof to treat a disease or condition. Accordingly, in some embodiments, an RLR agonist or RIG-VLP of the present disclosure is administered to a patent in need thereof to induce or increase T-cell activation, enhance T cell proliferation, induce the production and/or secretion of IFN, and/or induce a cytolytic T cell response.

While the present disclosure has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the disclosure. In addition, many modifications may be made to adapt a particular situation, material, composition

of matter, process, process step or steps, to the objective, spirit and scope of the present disclosure. All such modifications are intended to be within the scope of the disclosure.

**Definitions**

Terms used in the claims and specification are defined as set forth below unless otherwise specified. In the case of direct conflict with a term used in a parent provisional patent application, the term used in the instant application shall control.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

*About*: As used herein, the term "about" (alternatively “approximately”) will be understood by persons of ordinary skill and will vary to some extent depending on the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill given the context in which it is used, "about" will mean up to plus or minus 10% of the particular value.

*Agonist*: As used herein, the term “agonist” is used in its broadest sense and encompasses any molecule or compound that partially or fully promotes, induces, increases, and/or activates a biological activity of a native polypeptide disclosed herein. Agonist molecules according to the disclosure may include nucleic acids (e.g., oligonucleotides, polynucleotides), antibodies or antigen-binding fragments, fragments or amino acid sequence variants of native polypeptides, peptides, oligonucleotides, lipids, carbohydrates, and small organic molecules. In some embodiments, activation in the presence of the agonist is observed in a dose-dependent manner. In some embodiments, the measured signal (e.g., biological activity) is at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100% higher than the signal measured with a negative control under comparable conditions. Also disclosed herein, are methods of identifying agonists suitable for use in the methods of the disclosure. For example, these methods include, but are not limited to, binding assays such as enzyme-linked immuno-absorbent assay (ELISA), Forte Bio© systems, fluorescence polarization (FP) assay, and

radioimmunoassay (RIA). These assays determine the ability of an agonist to bind the polypeptide of interest (e.g., a receptor or ligand) and therefore indicate the ability of the agonist to promote, increase or activate the activity of the polypeptide. Efficacy of an agonist can also be determined using functional assays, such as the ability of an agonist to activate or promote the function of the polypeptide. For example, a functional assay may comprise contacting a polypeptide with a candidate agonist molecule and measuring a detectable change in one or more biological activities normally associated with the polypeptide. The potency of an agonist is usually defined by its $EC_{50}$ value (concentration required to activate 50% of the agonist response). The lower the $EC_{50}$ value the greater the potency of the agonist and the lower the concentration that is required to activate the maximum biological response.

*Ameliorating*: As used herein, the term "ameliorating" refers to any therapeutically beneficial result in the treatment of a disease state, e.g., cancer, including prophylaxis, lessening in the severity or progression, remission, or cure thereof.

*Amino acid*: As used herein, the term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, $\gamma$-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an a carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

Amino acids can be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, can be referred to by their commonly accepted single-letter codes.

*Amino acid substitution*: As used herein, an "amino acid substitution" refers to the replacement of at least one existing amino acid residue in a predetermined amino acid sequence

(an amino acid sequence of a starting polypeptide) with a second, different "replacement" amino acid residue. An "amino acid insertion" refers to the incorporation of at least one additional amino acid into a predetermined amino acid sequence. While the insertion will usually consist of the insertion of one or two amino acid residues, larger "peptide insertions," can also be made, e.g. insertion of about three to about five or even up to about ten, fifteen, or twenty amino acid residues. The inserted residue(s) may be naturally occurring or non- naturally occurring as disclosed above. An "amino acid deletion" refers to the removal of at least one amino acid residue from a predetermined amino acid sequence.

*Antigen*: As used herein, the term "antigen" refers to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a T helper cell epitope (Th cell epitope) and is given in adjuvant. An antigen can have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

*Antigenic determinant*: As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B- or T-lymphocytes. B-lymphocytes respond to foreign antigenic determinants via antibody production, whereas T-lymphocytes are the mediator of cellular immunity. Thus, antigenic determinants or epitopes are those parts of an antigen that are recognized by antibodies, or in the context of an MHC, by T-cell receptor*s*.

*Association*: As used herein, the term "association" as it applies to the first and second attachment sites, refers to the binding of the first and second attachment sites that is preferably by way of at least one non-peptide bond. The nature of the association may be covalent, ionic, hydrophobic, polar or any combination thereof, preferably the nature of the association is covalent, and again more preferably the association is through at least one, preferably one, non-peptide bond. As used herein, the term "association" as it applies to the first and second attachment sites, not

only encompass the direct binding or association of the first and second attachment site forming the compositions of the invention but also, alternatively and preferably, the indirect association or binding of the first and second attachment site leading to the compositions of the invention, and hereby typically and preferably by using a heterobifunctional cross-linker.

*First attachment site*: As used herein, the phrase "first attachment site" refers to an element of non-natural or natural origin, typically and preferably being comprised by the virus-like particle, to which the second attachment site typically and preferably being comprised by the antigen or antigenic determinant may associate. The first attachment site may be a protein, a polypeptide, an amino acid, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. The first attachment site is located, typically and preferably on the surface, of the virus-like particle. Multiple first attachment sites are present on the surface of virus-like particle typically in a repetitive configuration. Preferably, the first attachment site is an amino acid or a chemically reactive group thereof.

*Second attachment site*: As used herein, the phrase "second attachment site" refers to an element associated with, typically and preferably being comprised by, the antigen or antigenic determinant to which the first attachment site located on the surface of the virus-like particle may associate. The second attachment site of the antigen or antigenic determinant may be a protein, a polypeptide, a peptide, a sugar, a polynucleotide, a natural or synthetic polymer, a secondary metabolite or compound (biotin, fluorescein, retinol, digoxigenin, metal ions, phenylmethylsulfonylfluoride), or a combination thereof, or a chemically reactive group thereof. At least one second attachment site is present on the antigen or antigenic determinant. The term "antigen or antigenic determinant with at least one second attachment site" refers, therefore, to an antigen or antigenic construct comprising at least the antigen or antigenic determinant and the second attachment site. However, in particular for a second attachment site, which is of non-natural origin, i.e. not naturally occurring within the antigen or antigenic determinant, these antigen or antigenic constructs comprise an "amino acid linker".

*Base Composition*: As used herein, the term “base composition” refers to the proportion of the total nucleotides of a nucleic acid (e.g., an RNA) consisting of guanine (or hypoxanthine) + cytosine and/or uracil (or thymine) + adenine nucleobases.

*Base Pair*: As used herein, the term "base pair" refers to two nucleobases on opposite complementary polynucleotide strands, or regions of the same strand, that interact via the formation of specific hydrogen bonds. As used herein, the term "Watson-Crick base pairing", used interchangeably with "complementary base pairing", refers to a set of base pairing rules, wherein a purine always binds with a pyrimidine such that the nucleobase adenine (A) forms a complementary base pair with thymine (T) and guanine (G) forms a complementary base pair with cytosine (C) in DNA molecules. In RNA molecules, thymine is replaced by uracil (U), which, similar to thymine (T), forms a complementary base pair with adenine (A). The complementary base pairs are bound together by hydrogen bonds and the number of hydrogen bonds differs between base pairs. As in known in the art, guanine (G)-cytosine (C) base pairs are bound by three (3) hydrogen bonds and adenine (A)-thymine (T) or uracil (U) base pairs are bound by two (2) hydrogen bonds.

Base pairing interactions that do not follow these rules can occur in natural, non-natural, and synthetic nucleic acids and are referred to herein as "non-Watson-Crick base pairing" or alternatively "non-canonical base pairing". A "wobble base pair" is a pairing between two nucleobases in RNA molecules that does not follow Watson-Crick base pair rules. For example, inosine is a nucleoside that is structurally similar to guanosine, but is missing the 2-amino group. Inosine is able to form two hydrogen bonds with each of the four natural nucleobases (Oda et al., (1991) Nucleic Acids Res 19:5263-5267) and it is often used by researchers as a "universal" base, meaning that it can base pair with all the naturally-occurring or canonical bases. The four main wobble base pairs are the guanine-uracil (G-U) base pair, the hypoxanthine-uracil (I-U) base pair, the hypoxanthine-adenine (I-A) base pair, and the hypoxanthine-cytosine (I-C) base pair. In order to maintain consistency of nucleic acid nomenclature, "I" is used for hypoxanthine because hypoxanthine is the nucleobase of inosine; nomenclature otherwise follows the names of nucleobases and their corresponding nucleosides (e.g., "G" for both guanine and guanosine – as well as for deoxyguanosine). The thermodynamic stability of a wobble base pair is comparable to that of a Watson-Crick base pair. Wobble base pairs play a role in the formation of secondary structure in RNA molecules.

In one aspect, the disclosure provides synthetic RNA molecules that agonize or activate one or more RIG-I-like receptors (RLRs), wherein inosine can only be inserted at positions where

it will base pair with cytidine (I-C base pair); that is, inosine can be substituted for guanosine but cannot be substituted for the other nucleosides.

*Biologically active*: As used herein, the phrase "biologically active" refers to a characteristic of any substance that has activity in a biological system and/or organism. For instance, a substance that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active and thus have "biological activity". In particular embodiments, where a nucleic acid is biologically active, a portion of that nucleic acid that shares at least one biological activity of the whole nucleic acid is typically referred to as a "biologically active" portion.

*Bound*: As used herein, the term "bound" refers to binding that may be covalent, e.g., by chemically coupling, or non-covalent, e.g., ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphodiester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term "bound" is broader than and includes terms such as "coupled", "fused", "associated" and "attached". Moreover, with respect to the RLR agonist being bound to the virus-like particle the term "bound" also includes the enclosement, or partial enclosement, of the RLR agonist. Therefore, with respect to the RLR agonist being bound to the virus-like particle the term "bound" is broader than and includes terms such as "coupled," "fused," "enclosed", "packaged" and "attached." For example, the RLR agonist can be enclosed by the VLP without the existence of an actual binding, neither covalently nor non-covalently.

*Coat Protein*: As used herein, the term "coat protein(s)" refers to the protein(s) of a bacteriophage or a RNA-phage capable of being incorporated within the capsid assembly of the bacteriophage or the RNA-phage. However, when referring to the specific gene product of the coat protein gene of RNA-phages the term "CP" is used. For example, the specific gene product of the coat protein gene of RNA-phage Qβ is referred to as "Qβ CP", whereas the "coat proteins" of bacteriophage Qβ comprise the "Qβ CP" as well as the A1 protein. The capsid of bacteriophage Qβ is composed mainly of the Qβ CP, with a minor content of the A1 protein. Likewise, the VLP Qβ coat protein contains mainly Qβ CP, with a minor content of A1 protein.

*Covalently linked*: As used herein, the term "covalently linked" (alternatively "conjugated", "linked," "attached," "fused", or "tethered"), when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, by

whatever means including chemical conjugation, recombinant techniques or enzymatic activity, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions.

*Complementary*: As used herein, the term "complementary" or "complementarity" refers to a relationship between the sequence of nucleotides comprising two polynucleotide strands, or regions of the same polynucleotide strand, and the formation of a duplex comprising the strands or regions, wherein the extent of consecutive base pairing between the two strands or regions is sufficient for the generation of a duplex structure. It is known that adenine (A) forms specific hydrogen bonds, or "base pairs", with thymine (T) or uracil (U). Similarly, it is known that a cytosine (C) base pairs with guanine (G). It is also known that non-canonical nucleobases (e.g., inosine) can hydrogen bond with natural bases. A sequence of nucleotides comprising a first strand of a polynucleotide, or a region, portion or fragment thereof, is said to be "sufficiently complementary" to a sequence of nucleotides comprising a second strand of the same or a different nucleic acid, or a region, portion, or fragment thereof, if, when the first and second strands are arranged in an antiparallel fashion, the extent of base pairing between the two strands maintains the duplex structure under the conditions in which the duplex structure is used (e.g., physiological conditions in a cell). It should be understood that complementary strands or regions of polynucleotides can include some base pairs that are non-complementary. Complementarity may be "partial," in which only some of the nucleobases comprising the polynucleotide are matched according to base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. Although the degree of complementarity between polynucleotide strands or regions has significant effects on the efficiency and strength of hybridization between the strands or regions, it is not required for two complementary polynucleotides to base pair at every nucleotide position. In some embodiments, a first polynucleotide is 100% or "fully" complementary to a second polynucleotide and thus forms a base pair at every nucleotide position. In some embodiments, a first polynucleotide is not 100% complementary (e.g., is 90%, or 80% or 70% complementary) and contains mismatched nucleotides at one or more nucleotide positions. While perfect complementarity is often desired, some embodiments can include one or more but preferably 6, 5, 4, 3, 2, or 1 mismatches.

*Contacting*: As used herein, the term "contacting" means establishing a physical connection between two or more entities. For example, contacting a cell with an agent (e.g. an RNA, a lipid nanoparticle composition, or other pharmaceutical composition of the disclosure) means that the cell and the agent are made to share a physical connection. Methods of contacting cells with external entities both in vivo, in vitro, and ex vivo are well known in the biological arts. In exemplary embodiments of the disclosure, the step of contacting a mammalian cell with a composition (e.g., an isolated RNA, nanoparticle, or pharmaceutical composition of the disclosure) is performed in vivo. For example, contacting a lipid nanoparticle composition and a cell (for example, a mammalian cell) which may be disposed within an organism (e.g., a mammal) may be performed by any suitable administration route (e.g., parenteral administration to the organism, including intravenous, intramuscular, intradermal, and subcutaneous administration). For a cell present in vitro, a composition (e.g., a lipid nanoparticle or an isolated RNA) and a cell may be contacted, for example, by adding the composition to the culture medium of the cell and may involve or result in transfection. Moreover, more than one cell may be contacted by an agent.

*Coupled*: As used herein, the term "coupled" refers to attachment by covalent bonds or by strong non-covalent interactions. With respect to the coupling of the antigen to the virus-like particle the term "coupled" preferably refers to attachment by covalent bonds. Moreover, with respect to the coupling of the antigen to the virus-like particle the term "coupled" preferably refers to association and attachment, respectively, by at least one non-peptide bond. Any method normally used by those skilled in the art for the coupling of biologically active materials can be used in the present invention.

*Denaturation*: As used herein, the term "denaturation" refers to the process by which the hydrogen bonding between base paired nucleotides in a nucleic acid is disrupted, resulting in the loss of secondary and/or tertiary nucleic acid structure (e.g. the separation of previously annealed strands). Denaturation can occur by the application of an external substance, energy, or biochemical process to a nucleic acid.

*Antigen presenting cell*: The term "antigen presenting cell" or "APC" is a cell that displays foreign antigen complexed with MHC on its surface. T cells recognize this complex using T cell receptor (TCR). Examples of APCs include, but are not limited to, dendritic cells (DCs), peripheral blood mononuclear cells (PBMC), monocytes (such as THP-1), B lymphoblastoid cells

(such as C1R.A2, 1518 B-LCL) and monocyte-derived dendritic cells (DCs). Some APCs internalize antigens either by phagocytosis or by receptor-mediated endocytosis.

*Apoptosis*: As used herein, the term "apoptosis" refers to the process of programmed cell death that occurs in multicellular organisms (e.g. humans). The highly-regulated biochemical and molecular events that result in apoptosis can lead to observable and characteristic morphological changes to a cell, including membrane blebbing, cell volume shrinkage, chromosomal DNA condensation and fragmentation, and mRNA decay. A common method to identify cells, including T cells, undergoing apoptosis is to expose cells to a fluorophore-conjugated protein (Annexin V). Annexin V is commonly used to detect apoptotic cells by its ability to bind to phosphatidylserine on the outer leaflet of the plasma membrane, which is an early indicator that the cell is undergoing the process of apoptosis.

*Blunt-end:* As used herein, the term “blunt-end” “blunt-ended” refers to the structure of an end of a duplexed or double-stranded nucleic acid, wherein both complementary strands comprising the duplex terminate, at least at one end, in a base pair. Hence, neither strand comprising the duplex extends further from the end than the other.

*Cancer antigen*: As used herein, "cancer antigen" refers to (i) tumor- specific antigens, such as neoantigens, (ii) tumor- associated antigens, (iii) cells that express tumor- specific antigens, (iv) cells that express tumor- associated antigens, (v) embryonic antigens on tumors, (vi) autologous tumor cells, (vii) tumor- specific membrane antigens, (viii) tumor- associated membrane antigens, (ix) growth factor receptors, (x) growth factor ligands, and (xi) any other type of antigen or antigen-presenting cell or material that is associated with a cancer.

*Carcinoma*: As used herein, the term "carcinoma" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. The RIG-I-like receptor (RLR) agonists described herein can be used to treat patients who have, who are suspected of having, or who may be at high risk for developing any type of cancer, including renal carcinoma or melanoma. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An

"adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

*Cytotoxic T lymphocyte (CTL) response:* As used herein, the term "cytotoxic T lymphocyte (CTL) response" refers to an immune response induced by cytotoxic T cells. CTL responses are mediated primarily by CD8+ T cells.

*Duplex*: As used herein, the term "duplex" refers to a structure formed by complementary strands of a double-stranded polynucleotide, or complementary regions of a single-stranded polynucleotide that folds back on itself. The duplex structure of a nucleic acid arises as a consequence of complementary nucleotide sequences being bound together, or hybridizing, by base pairing interactions.

*$EC_{50}$*: As used herein, the term "$EC_{50}$" refers to the concentration of an agonist which induces a response, either in an in vitro or an in vivo assay, which is 50% of the maximal response, i.e., halfway between the maximal response and the baseline.

*Effective dose*: As used herein, the term "effective dose" or "effective dosage" is defined as an amount sufficient to achieve or at least partially achieve the desired effect.

*Fusion*: As used herein, the term "fusion" refers to the combination of amino acid sequences of different origin in one polypeptide chain by in-frame combination of their coding nucleotide sequences. The term "fusion" explicitly encompasses internal fusions, i.e., insertion of sequences of different origin within a polypeptide chain, in addition to fusion to one of its termini.

*Hairpin RNA*: As used herein, the term "hairpin RNA" or "RNA hairpin" refers to a self-complementary RNA comprising a double-stranded RNA (dsRNA) stem comprised of complementary nucleotide strands that base pair to form a duplex that terminates at one end in a nucleotide linker comprising a loop of unpaired nucleotides (e.g., a tetraloop) comprising unpaired nucleotides or in a non-nucleotide linker comprising a flexible chemical moiety (e.g., ethylene glycol), either of which connects the complementary nucleotide strands. RNA hairpins may differ in the length of the stem, the size and/or composition of the loop and/or linker, the number of base pair mismatches within the stem, and in the actual nucleotide sequence. RNA hairpins may provide one or more functions, including, but not limited to, guiding the overall folding of an RNA molecule comprising the hairpin, determining interactions in a ribozyme, protecting messenger RNA (e.g., mRNA) from degradation, serving as a recognition motif or structure for RNA binding proteins and acting as a substrate for enzymatic reactions. Further description of RNA hairpin

structures and functions can be found in Svoboda and Di Cara (2006) Cell Mol Life Sci 63(7-8):901-908, and references contained therein. In some embodiments, the stem regions of the hairpin RNAs comprising the RLR agonists provided by the disclosure terminate in a blunt end with a 5' triphosphate or diphosphate.

*Improved biological activity*: As used herein, a composition which "improves" a biological activity refers to a substance in which an biological activity is observed that is greater or intensified or deviated in any way with the addition of the composition when compared to the same biological activity measured without the addition of the composition. For example, the amount of cytokines secreted can be measured, e.g. using an ELISA assay, from samples treated with and without the composition. The amount of the composition at which the cytokine secretion is enhanced as compared to cytokine secretion without the composition is said to be an amount sufficient to improve the biological activity. In one embodiment, the biological activity is improved by a factor of at least about 2, more preferably by a factor of about 3 or more. The lytic activity of cytotoxic T cells may also be altered.

*In need*: As used herein, a subject "in need of prevention," "in need of treatment," or "in need thereof," refers to one, who by the judgment of an appropriate medical practitioner (e.g., a doctor, a nurse, or a nurse practitioner in the case of humans; a veterinarian in the case of non-human mammals), would reasonably benefit from a given treatment (such as treatment with a composition comprising a RIG-I-like receptor agonist).

*Linker*: As used herein, the term "linker" (alternatively "tether" or "spacer") refers to a moiety that covalently connects, attaches or couples two polynucleotide strands or regions together. As used herein, a linker comprising nucleotides is referred to as a "nucleotide linker" (e.g. a tetraloop). As used herein, the term "non-nucleotide linker" refers to a linker comprising a chemical moiety and that does not comprise a nucleotide. Non-limiting examples of non-nucleotide linkers include linkers comprising ethylene glycol (e.g. hexaethylene glycol), alkyl chains (e.g. C9 alkyl linker), and stilbene diether. Further description of linkers can be found in Paredes et al., (2011) Methods 54:251-259, which is incorporated herein by reference in its entirety.

*LGP2*: As used herein, the term "LGP2" refers to the Laboratory of Genetics and Physiology 2 polypeptide, a specific member of the RIG-I-like receptor family and is encoded by the *DHX58* gene in humans. Alternative names and acronyms for LGP2 in the art include DHX58,

D11LGP2, D11Igp2e, and RLR-3. An exemplary amino acid sequence of full-length human LGP2 is set forth in Table 4 (SEQ ID NO: 100) and here:

MELRSYQWEVIMPALEGKNIIIWLPTGAGKTRAAAYVAKRHLETVDGAKVVVL
VNRVHLVTQHGEEFRRMLDGRWTVTTLSGDMGPRAGFGHLARCHDLLICTAELLQMA
LTSPEEEEHVELTVFSLIVVDECHHTHKDTVYNVIMSQYLELKLQRAQPLPQVLGLTASP
GTGGASKLDGAINHVLQLCANLDTWCIMSPQNCCPQLQEHSQQPCKQYNLCHRRSQDP
FGDLLKKLMDQIHDHLEMPELSRKFGTQMYEQQVVKLSEAAALAGLQEQRVYALHLR
RYNDALLIHDTVRAVDALAALQDFYHREHVTKTQILCAERRLLALFDDRKNELAHLAT
HGPENPKLEMLEKILQRQFSSSNSPRGIIFTRTRQSAHSLLLWLQQQQGLQTVDIRAQLLI
GAGNSSQSTHMTQRDQQEVIQKFQDGTLNLLVATSVAEEGLDIPHCNVVVRYGLLTNEI
SMVQARGRARADQSVYAFVATEGSRELKRELINEALETLMEQAVAAVQKMDQAEYQA
KIRDLQQAALTKRAAQAAQRENQRQQFPVEHVQLLCINCMVAVGHGSDLRKVEGTHH
VNVNPNFSNYYNVSRDPVVINKVFKDWKPGGVISCRNCGEVWGLQMIYKSVKLPVLK
VRSMLLETPQGRIQAKKWSRVPFSVPDFDFLQHCAENLSDLSLD

(NCBP Accession Number: NP_077024.2)

*Local administration*: As used herein, "local administration" or "local delivery," refers to delivery that does not rely upon transport of the composition or agent to its intended target tissue or site via the vascular system. For example, the composition may be delivered by injection or implantation of the composition or agent or by injection or implantation of a device containing the composition or agent. Following local administration in the vicinity of a target tissue or site, the composition or agent, or one or more components thereof, may diffuse to the intended target tissue or site.

*MDA5*: As used herein, the term "MDA5" refers to the Melanoma Differentiation-Associated Protein 5 polypeptide, a specific member of the RIG-I-like receptor family and is encoded by the *IFIH1* gene in humans. Alternative names and acronyms for MDA5 in the art include AGS7, Hlcd, IDDM19, MDA-5, RLR-2, SGMRT1, and interferon induced with helicase C domain 1. An exemplary amino acid sequence of full-length human MDA5 is set forth in Table 4 (SEQ ID NO: 99) and here:

MSNGYSTDENFRYLISCFRARVKMYIQVEPVLDYLTFLPAEVKEQIQRTVATSGN
MQAVELLLSTLEKGVWHLGWTREFVEALRRTGSPLAARYMNPELTDLPSPSFENAHDE
YLQLLNLLQPTLVDKLLVRDVLDKCMEEELLTIEDRNRIAAAENNGNESGVRELLKRIV

QKENWFSAFLNVLRQTGNNELVQELTGSDCSESNAEIENLSQVDGPQVEEQLLSTTVQP
NLEKEVWGMENNSSESSFADSSVVSESDTSLAEGSVSCLDESLGHNSNMGSDSGTMGS
DSDEENVAARASPEPELQLRPYQMEVAQPALEGKNIIICLPTGSGKTRVAVYIAKDHLDK
KKKASEPGKVIVLVNKVLLVEQLFRKEFQPFLKKWYRVIGLSGDTQLKISFPEVVKSCDI
IISTAQILENSLLNLENGEDAGVQLSDFSLIIIDECHHTNKEAVYNNIMRHYLMQKLKNNR
LKKENKPVIPLPQILGLTASPGVGGATKQAKAEEHILKLCANLDAFTIKTVKENLDQLKN
QIQEPCKKFAIADATREDPFKEKLLEIMTRIQTYCQMSPMSDFGTQPYEQWAIQMEKKA
AKEGNRKERVCAEHLRKYNEALQINDTIRMIDAYTHLETFYNEEKDKKFAVIEDDSDEG
GDDEYCDGDEDEDDLKKPLKLDETDRFLMTLFFENNKMLKRLAENPEYENEKLTKLRN
TIMEQYTRTEESARGIIFTKTRQSAYALSQWITENEKFAEVGVKAHHLIGAGHSSEFKPM
TQNEQKEVISKFRTGKINLLIATTVAEEGLDIKECNIVIRYGLVTNEIAMVQARGRARAD
ESTYVLVAHSGSGVIEHETVNDFREKMMYKAIHCVQNMKPEEYAHKILELQMQSIMEK
KMKTKRNIAKHYKNNPSLITFLCKNCSVLACSGEDIHVIEKMHHVNMTPEFKELYIVRE
NKALQKKCADYQINGEIICKCGQAWGTMMVHKGLDLPCLKIRNFVVVFKNNSTKKQY
KKWVELPITFPNLDYSECCLFSDED

(NCBI Accession Number: NP_071451.2)

*Modified*: As used herein "modified" or "modification" refers to a changed state or change in structure resulting from a modification of a polynucleotide, e.g., RNA. Polynucleotides may be modified in various ways including chemically, structurally, and/or functionally. For example, the RNA molecules of the present disclosure may be modified by the incorporation of a non-natural base or a sequence motif, comprising a functional sequence or secondary structure, that provides a biological activity. In one embodiment, the RNA is modified by the introduction of non-natural or chemically-modified bases, nucleosides and/or nucleotides, e.g., as it relates to the natural ribonucleotides A, U, G, and C.

*Naturally-occurring*: As used herein, the term "naturally-occurring" as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence, or components thereof such as amino acids or nucleotides, that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

*Nucleic acid*: As used herein, the term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers or oligomers thereof in either single- or double- stranded form.

Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Polymers of nucleotides are referred to as "polynucleotides". Exemplary nucleic acids or polynucleotides of the disclosure include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), DNA-RNA hybrids, RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, RNAs that induce triple helix formation, threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β-D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2'-amino-LNA having a 2'-amino functionalization, and 2'-amino-α-LNA having a 2'-amino functionalization) or hybrids thereof.

Polynucleotides used herein can be composed of any polyribonucleotide or polydeoxribonucleotide, which can be unmodified RNA or DNA or modified RNA or DNA. For example, polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double- stranded regions, single- and double- stranded RNA, and RNA that is mixture of single- and double- stranded regions, hybrid molecules comprising DNA and RNA that can be single- stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the polynucleotide can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. A polynucleotide can also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases. "Modified nucleosides" include, for example, as inosine and thymine, when the latter is found in or comprises RNA. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

*Nucleic Acid Structure*: As used herein, the term "nucleic acid structure" refers to the arrangement or organization of atoms, chemical constituents, elements, motifs, and/or sequence of nucleobases that comprise a nucleic acid (e.g. an RNA) and/or can refer to the two-dimensional or three-dimensional state of a nucleic acid. Accordingly, the term "RNA structure" refers to the arrangement or organization of atoms, chemical constituents, elements, motifs, and/or sequence of nucleobases comprising an RNA molecule (e.g. an mRNA) and/or can refer to the two-dimensional and/or three dimensional state of an RNA molecule. Nucleic acid structure can be further

demarcated into four organizational categories referred to herein as "molecular structure", "primary structure", "secondary structure", and "tertiary structure" based on increasing organizational complexity.

*Nucleobase*: As used herein, the term "nucleobase" (alternatively "nucleotide base" or "nitrogenous base") refers to a purine or pyrimidine heterocyclic compound found in nucleic acids, including any derivatives or analogs of the naturally occurring purines and pyrimidines that confer improved properties (e.g. binding affinity, nuclease resistance, chemical stability) to a nucleic acid or a portion or segment thereof. Adenine, cytosine, guanine, thymine, and uracil are the primary or canonical nucleobases predominately found in natural nucleic acids. Other natural, non-natural, non-canonical and/or synthetic nucleobases, can be incorporated into nucleic acids, such as those disclosed herein.

*Nucleoside/Nucleotide*: As used herein, the term "nucleoside" refers to a compound containing a sugar molecule (e.g., a ribose in RNA or a deoxyribose in DNA), or derivative or analog thereof, covalently linked to a nucleobase (e.g., a purine or pyrimidine), or a derivative or analog thereof (also referred to herein as "nucleobase"). As used herein, the term "nucleotide" refers to a nucleoside covalently linked to a phosphate group. As used herein, the term "ribonucleoside" refers to a nucleoside that comprise a ribose and a nucleobase (e.g., adenosine (A), cytidine (C), guanosine (G), 5-methyluridine ($m^5U$), uridine (U), or inosine (I)).

*Operably linked*: As used herein, a nucleic acid, or fragment or portion thereof, such as a polynucleotide or oligonucleotide is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence, or fragment or portion thereof.

*Ordered and repetitive antigen or antigenic determinant array*: As used herein, the term "ordered and repetitive antigen or antigenic determinant array" generally refers to a repeating pattern of antigen or antigenic determinant, characterized by a typically and preferably uniform spacial arrangement of the antigens or antigenic determinants with respect to the core particle and virus-like particle, respectively. In some embodiments, the repeating pattern may be a geometric pattern. Typical examples of suitable ordered and repetitive antigen or antigenic determinant arrays are those which possess strictly repetitive paracrystalline orders of antigens or antigenic determinants, preferably with spacings of 0.5 to 30 nanometers, more preferably 3 to 15 nanometers, even more preferably 3 to 8 nanometers.

*Packaged*: As used herein, the term "packaged" refers to the state of an RLR agonist in relation to the VLP. The term "packaged" as used herein includes binding that may be covalent, e.g., by chemically coupling, or non-covalent, e.g., ionic interactions, hydrophobic interactions, hydrogen bonds, etc. Covalent bonds can be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term also includes the enclosement, or partial enclosement, of a substance. The term "packaged" includes terms such as "coupled, "enclosed" and "attached." For example, the RLR agonist can be enclosed by the VLP without the existence of an actual binding, neither covalently nor non-covalently. In some embodiments, the term "packaged" indicates that the nucleic acid in a packaged state is not accessible to DNAse or RNAse hydrolysis. In some embodiments, the RLR agonist is packaged inside the VLP capsids, most preferably in a non-covalent manner.

*Polynucleotide/oligonucleotide*: As used herein, the terms “polynucleotide” and “oligonucleotide” are used interchangeably and refer to a single-stranded or double-stranded polymer or oligomer of nucleotides or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms “polynucleotide” and “oligonucleotide” also includes polymers and oligomers comprising non-naturally occurring bases, sugars and intersugar (backbone) linkages, or portions thereof, which function similarly. Polynucleotides are not limited to any particular length of nucleotide sequence, as the term “polynucleotides” encompasses polymeric forms of nucleotides of any length. Short polynucleotides are typically referred to in the art as “oligonucleotides”. In the context of the present disclosure, such modified or substituted polynucleotides and oligonucleotides are often used over native forms because the modification increases one or more desirable or beneficial biological properties or activities including, but not limited to, increased cytokine production, enhanced cellular uptake and/or increased stability in the presence of nucleases. In some embodiments, the agonists of the disclosure comprise polynucleotides and oligonucleotides that contain at least one region of modified nucleotides that confers one or more beneficial properties or increases biological activity (e.g., increased nuclease resistance, increased uptake into cells, increased duplex stability, increased binding affinity to a target polypeptide).

*Palindromic sequence*: As used herein, the term “palindromic sequence” (alternatively “palindrome”) refers to a sequence of nucleotides that is self-complementary; wherein the sequence of nucleotides in the 5' to 3' direction is the same as the sequence of nucleotides

comprising the complementary strand, when read in the 5' to 3'. For example, the sequence 5'-ACCTAGGT-3' is a palindromic sequence because its complementary sequence, 3'-TGGATCCA-5', when read in the 5' to 3' direction, is the same as the original sequence. In contrast, the sequence 5'-AGTGGCTG-3' is not a palindromic sequence because its complementary sequence, 3'-TCACCGAC-5', when read in the 5' to 3' direction, is not the same as the original sequence.

In one embodiment, the agonist is comprised of a first oligonucleotide, wherein the sequence of the first oligonucleotide is a palindromic sequence. In another embodiment, the agonist is comprised of a first oligonucleotide, wherein the first oligonucleotide comprises a palindromic sequence.

In one embodiment, palindromic sequences in oligonucleotides of the invention include both the 5' end of the oligonucleotide and the 3' end of the oligonucleotide, thus forming a blunt end. In one embodiment of the invention the oligonucleotide comprises a single palindromic sequence and in another embodiment of the invention the oligonucleotide comprises two complementary palindromes interrupted by an intervening sequence, spacer, or linker that connects the 2 palindromes within 1 or 2 different oligonucleotides so as to form a hairpin duplex with a blunt end.

*Parenteral administration*: As used herein, "parenteral administration," "administered parenterally," and other grammatically equivalent phrases, refer to modes of administration other than enteral and topical administration, usually by injection, and include, without limitation, intravenous, intranasal, intraocular, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural, intracerebral, intracranial, intracarotid and intrasternal injection and infusion.

*Patient*: As used herein, the term "patient" includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

*Percent identity*: As used herein, the term "percent identity," in the context of two or more nucleic acid or polypeptide sequences, refers to two or more sequences or subsequences that have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below (e.g., BLASTP and BLASTN or other algorithms available to persons

of skill) or by visual inspection. Depending on the application, the "percent identity" can exist over a region of the sequence being compared, e.g., over a functional domain, or, alternatively, exist over the full length of the two sequences to be compared. For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. The percent identity between two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by visual inspection (see generally Ausubel et al., infra).

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul et al., J. Mol. Biol. 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website. The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In

addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol. Biol. (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

The nucleic acid and protein sequences of the present disclosure can further be used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

*Pharmaceutically acceptable*: As used herein, the term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues, organs, and/or bodily fluids of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

*Pharmaceutically acceptable carrier*: As used herein, the term "pharmaceutically acceptable carrier" refers to, and includes, any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. The compositions can include a pharmaceutically acceptable salt, e.g., an acid addition salt or a base addition salt (see, e.g., Berge et al. (1977) J Pharm Sci 66:1-19).

*Phosphate*: The term "phosphate" as used herein means a salt or ester of phosphoric acid. Polyphosphates are salts or esters of polymeric oxyanions formed from tetrahedral $PO_4$ (phosphate) structural units linked together by sharing oxygen atoms. As used herein, the term

"diphosphate" refers to a polyphosphate comprising two phosphate structural units. As used herein, the term "triphosphate" refers to a polyphosphate comprising three phosphate structural units. In some embodiments, the disclosure provides a RIG-I-like receptor agonist comprising a diphosphate moiety, or a derivative or analog thereof, linked to the 5' terminus. In some embodiments, the disclosure provides a RIG-I-like receptor agonist comprising a triphosphate moiety, or a derivative or analog thereof, linked to the 5' terminus. In some embodiments, the derivative or analog thereof is a phosphate bioisostere.

*Phosphate bioisostere:* As used herein, the term "phosphate bioisostere" (alternatively "phosphate mimic") refers to chemical substituents or groups with similar physical or chemical properties to phosphate and which produce broadly similar biological properties to phosphate, including diphosphate and triphosphate moieties. In drug design, the purpose of exchanging one bioisostere for another is to enhance the desired biological or physical properties of a compound without making significant changes in chemical structure. The use of bioisosteres is widespread in drug development and is used, for example, to reduce toxicity, change bioavailability, or modify the activity or metabolism of the parental or lead compound (see e.g., Rye and Baell (2005) Curr Med Chem 12(26):3127-3141; Elliot et al., (2012) MedChemCom 3(7):735-751, which are incorporated herein by reference in their entirety).

*Polypeptide*: As used herein, the terms "polypeptide," "peptide", and "protein" are used interchangeably to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

*Preventing*: As used herein, the term "preventing" when used in relation to a condition, refers to administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition in a subject relative to a subject which does not receive the composition.

*Purified*: As used herein, the term "purified" or "isolated" as applied to any of the proteins (antibodies or fragments) described herein refers to a polypeptide that has been separated or purified from components (e.g., proteins or other naturally-occurring biological or organic molecules) which naturally accompany it, e.g., other proteins, lipids, and nucleic acid in a prokaryote expressing the proteins. Typically, a polypeptide is purified when it constitutes at least

60 (e.g., at least 65, 70, 75, 80, 85, 90, 92, 95, 97, or 99) %, by weight, of the total protein in a sample.

*Reference ligand*: As used herein, the term "reference ligand" (used interchangeably with "reference agonist") or "reference molecule" refers to a RIG-I-like receptor ligand and is used to establish a relationship between itself and one or more distinct RIG-I-like receptor ligands, wherein the relationship is the relative agonistic effect of the reference ligand and the one or more distinct RIG-I-like receptor ligands. As used herein, the term connotes a RIG-I-like receptor ligand or agonist that is useful in a test or assay, such as those described herein, (e.g., an IFN induction assay), as a competitor, wherein the assay is useful for the discovery, identification or development, of one or more distinct agonists that bind to RIG-I-like receptors.

*RIG-I*: As used herein, the term "RIG-I" refers to the Retinoic Acid-Inducible Gene I polypeptide, a specific member of the RIG-I-like receptor family and is encoded by the *DDX58* gene in humans. Alternative names and acronyms for RIG-I in the art include DEAD box polypeptide 58, RIGI, RLR-1, SGMRT2, and DEXD/H-box helicase 58. An exemplary amino acid sequence of full-length human RIG-I is set forth in Table 4 (SEQ ID NO: 98) and here:

MTTEQRRSLQAFQDYIRKTLDPTYILSYMAPWFREEEVQYIQAEKNNKGPMEAA
TLFLKFLLELQEEGWFRGFLDALDHAGYSGLYEAIESWDFKKIEKLEEYRLLLKRLQPEF
KTRIIPTDIISDLSECLINQECEEILQICSTKGMMAGAEKLVECLLRSDKENWPKTLKLALE
KERNKFSELWIVEKGIKDVETEDLEDKMETSDIQIFYQEDPECQNLSENSCPPSEVSDTNL
YSPFKPRNYQLELALPAMKGKNTIICAPTGCGKTFVSLLICEHHLKKFPQGQKGKVVFFA
NQIPVYEQQKSVFSKYFERHGYRVTGISGATAENVPVEQIVENNDIIILTPQILVNNLKKG
TIPSLSIFTLMIFDECHNTSKQHPYNMIMFNYLDQKLGGSSGPLPQVIGLTASVGVGDAK
NTDEALDYICKLCASLDASVIATVKHNLEELEQVVYKPQKFFRKVESRISDKFKYIIAQL
MRDTESLAKRICKDLENLSQIQNREFGTQKYEQWIVTVQKACMVFQMPDKDEESRICK
ALFLYTSHLRKYNDALIISEHARMKDALDYLKDFFSNVRAAGFDEIEQDLTQRFEEKLQE
LESVSRDPSNENPKLEDLCFILQEEYHLNPETITILFVKTRALVDALKNWIEGNPKLSFLK
PGILTGRGKTNQNTGMTLPAQKCILDAFKASGDHNILIATSVADEGIDIAQCNLVILYEY
VGNVIKMIQTRGRGRARGSKCFLLTSNAGVIEKEQINMYKEKMMNDSILRLQTWDEAV
FREKILHIQTHEKFIRDSQEKPKPVPDKENKKLLCRKCKALACYTADVRVIEECHYTVLG
DAFKECFVSRPHPKPKQFSSFEKRAKIFCARQNCSHDWGIHVKYKTFEIPVIKIESFVVED
IATGVQTLYSKWKDFHFEKIPFDPAEMSK

(NCBI Accession Number: NP_055129.2)

*RIG-I-like receptor*: As used herein, the term "RIG-I-like receptor" (abbreviate as "RLR") refers to any member of a family of DExD/H box RNA helicases that function as cytoplasmic pattern recognition sensors of pathogen-associated molecular patterns (PAMPs) typically found in viral RNA. Upon ligand binding, RLRs signal downstream transcription factor activation to drive type 1 interferon (IFN) production and antiviral gene expression that elicits an intracellular immune response to control virus infection. Three RLR members have been identified: RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation associated factor 5), and LGP2 (laboratory of genetics and physiology 2 and a homolog of mouse D11lgp2) (Loo and Gale (2011) Immunity 34(5):680-692).

*RIG-I-like receptor agonist*: As used herein, the term "RIG-I-like receptor agonist" (used interchangeably with the term "RLR agonist") refers to a nucleic acid (e.g., an RNA) that binds to RIG-I-like receptors (RLRs) and partially or fully promotes, induces, increases, and/or activates a biological activity, response, and/or downstream pathway(s) mediated by RLR signaling or other RLR-mediated function. Examples of RIG-I-receptor agonists are provided herein. In some embodiments, a RLR agonist is a single-stranded nucleic acid (e.g., RNA). In some embodiments, a RLR agonist is a double-stranded nucleic acid (e.g., RNA).

*Stable RNA secondary structure*: As used herein, the term "stable RNA secondary structure" refers to a structure, fold, or conformation adopted by an RNA molecule, or local segment or portion thereof, that is persistently maintained under physiological conditions and characterized by a low free energy state. Typical examples of stable RNA secondary structures include duplexes, hairpins, and stem-loops. Stable RNA secondary structures are known in the art to exhibit various biological activities. The term "stable" as used in reference to a polynucleotide duplex, means that the duplex remains hybridized, structured or annealed essentially exclusively in the form of a duplex under physiological conditions or under typical salt and temperature conditions used in nucleic acid diagnostic or therapeutic applications.

*Subject*: As used herein, the term "subject" includes any human or non-human animal. For example, the methods and compositions of the present invention can be used to treat a subject with an immune disorder. The term "non-human animal" includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc.

*T cell*: The term "T cell" refers to a type of white blood cell that can be distinguished from other white blood cells by the presence of a T cell receptor on the cell surface. There are several subsets of T cells, including, but not limited to, T helper cells (a.k.a. TH cells or CD4+ T cells) and subtypes, including TH1, TH2, TH3, TH17, TH9, and TFH cells, cytotoxic T cells (a.k.a TC cells, CD8+ T cells, cytotoxic T lymphocytes, T-killer cells, killer T cells), memory T cells and subtypes, including central memory T cells (TCM cells), effector memory T cells (TEM and TEMRA cells), and resident memory T cells (TRM cells), regulatory T cells (a.k.a. Treg cells or suppressor T cells) and subtypes, including CD4+ FOXP3+ Treg cells, CD4+FOXP3- Treg cells, Tr1 cells, Th3 cells, and Treg17 cells, natural killer T cells (a.k.a. NKT cells), mucosal associated invariant T cells (MAITs), and gamma delta T cells (γδ T cells), including Vγ9/Vδ2 T cells. Any one or more of the aforementioned or unmentioned T cells may be the target cell type for a method of use of the invention.

*T cell activation*: As used herein, the term "T cell activation" or "activation of T cells" refers to a cellular process in which mature T cells, which express antigen-specific T cell receptors on their surfaces, recognize their cognate antigens and respond by entering the cell cycle, secreting cytokines or lytic enzymes, and initiating or becoming competent to perform cell-based effector functions. T cell activation requires at least two signals to become fully activated. The first occurs after engagement of the T cell antigen-specific receptor (TCR) by the antigen-major histocompatibility complex (MHC), and the second by subsequent engagement of co-stimulatory molecules (e.g., CD28). These signals are transmitted to the nucleus and result in clonal expansion of T cells, upregulation of activation markers on the cell surface, differentiation into effector cells, induction of cytotoxicity or cytokine secretion, induction of apoptosis, or a combination thereof.

*T cell-mediated response*: As used herein, the term "T cell-mediated response" refers to any response mediated by T cells, including, but not limited to, effector T cells (e.g., CD8+ cells) and helper T cells (e.g., CD4+ cells). T cell mediated responses include, for example, T cell cytotoxicity and proliferation.

*Tetraloop:* As used herein, the term "tetraloop" refers to a type of four-base loop motif found in hairpin or stem-loop RNA secondary structures that cap duplexes at one end, linking the two strands comprising the duplex, and provide stability to the hairpin structure.

*Therapeutic agent*: As used herein, the term "therapeutic agent" refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

*Therapeutically effective amount*: As used herein, the terms "therapeutically effective amount" or "therapeutically effective dose," or similar terms used herein are intended to mean an amount of an agent (e.g., a synthetic RIG-I-like receptor agonist) that will elicit the desired biological or medical response, such as, for example, curing or at least partially arresting the condition or disease and its complications in a patient already suffering from the disease (e.g., an improvement in one or more symptoms of a cancer). Amounts effective for this use will depend on the severity of the disorder being treated and the general state of the patient's own immune system.

*Treat*: The terms "treat," "treating," and "treatment," as used herein, refer to therapeutic or preventative measures described herein. The methods of "treatment" employ administration to a subject, in need of such treatment, a human antibody of the present disclosure, for example, a subject in need of an enhanced immune response against a particular antigen or a subject who ultimately may acquire such a disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of the disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

*Tumor microenvironment*: As used herein, the term "tumor microenvironment" (alternatively "cancer microenvironment"; abbreviated TME) refers to the cellular environment or milieu in which the tumor or neoplasm exists, including surrounding blood vessels as well as non-cancerous cells including, but not limited to, immune cells, fibroblasts, bone marrow-derived inflammatory cells, and lymphocytes. Signaling molecules and the extracellular matrix also comprise the TME. The tumor and the surrounding microenvironment are closely related and interact constantly. Tumors can influence the microenvironment by releasing extracellular signals, promoting tumor angiogenesis and inducing peripheral immune tolerance, while the immune cells in the microenvironment can affect the growth and evolution of tumor cells.

*Virus-like particle (VLP)*: As used herein, the term "virus-like particle" or "VLP" refers to a structure resembling a virus particle but which has not been demonstrated to be pathogenic. Typically, a virus-like particle in accordance with the disclosure does not carry genetic information encoding for the proteins of the virus-like particle. In general, virus-like particles lack the viral

genome and, therefore, are noninfectious. Also, virus-like particles can often be produced in large quantities by heterologous expression and can be easily purified. Some virus-like particles may contain nucleic acid distinct from their genome. As indicated, a virus-like particle in accordance with the disclosure is non replicative and noninfectious since it lacks all or part of the viral genome, in particular the replicative and infectious components of the viral genome. A virus-like particle in accordance with the disclosure may contain nucleic acid distinct from their genome. In some embodiments, a virus-like particle in accordance with the present disclosure is a viral capsid such as the viral capsid of the corresponding virus, bacteriophage, or RNA-phage. The terms "viral capsid" or "capsid", as interchangeably used herein, refer to a macromolecular assembly composed of viral protein subunits. Typically and preferably, the viral protein subunits assemble into a viral capsid and capsid, respectively, having a structure with an inherent repetitive organization, wherein said structure is, typically, spherical or tubular. For example, the capsids of RNA-phages or HBcAg's have a spherical form of icosahedral symmetry. The term "capsid-like structure" as used herein, refers to a macromolecular assembly composed of viral protein subunits ressembling the capsid morphology in the above defined sense but deviating from the typical symmetrical assembly while maintaining a sufficient degree of order and repetitiveness.

*Virus-like particle of a bacteriophage*: As used herein, the term "virus-like particle of a bacteriophage" refers to a virus-like particle resembling the structure of a bacteriophage, being non replicative and noninfectious, and lacking at least the gene or genes encoding for the replication machinery of the bacteriophage, and typically also lacking the gene or genes encoding the protein or proteins responsible for viral attachment to or entry into the host. This definition should, however, also encompass virus-like particles of bacteriophages, in which the aforementioned gene or genes are still present but inactive, and, therefore, also leading to non-replicative and noninfectious virus-like particles of a bacteriophage.

The capsid structure formed from the self-assembly of 180 subunits of RNA phage coat protein and optionally containing host RNA is referred to as a "VLP of RNA phage coat protein". A specific example is the VLP of Qβ coat protein. In this particular case, the VLP of Qβ coat protein may either be assembled exclusively from Qβ CP subunits (generated by expression of a Qβ CP gene containing, for example, a TAA stop codon precluding any expression of the longer A1 protein through suppression, see Kozlovska, T. M., et al., Intervirology 39: 9-15 (1996)), or additionally contain A1 protein subunits in the capsid assembly.

*Viral particle*: The term "virus particle" as used herein refers to the morphological form of a virus. In some virus types it comprises a genome surrounded by a protein capsid; others have additional structures (e.g., envelopes, tails, etc.). Non-enveloped viral particles are made up of a proteinaceous capsid that surrounds and protects the viral genome. Enveloped viruses also have a capsid structure surrounding the genetic material of the virus but, in addition, have a lipid bilayer envelope that surrounds the capsid. In some embodiments, the VLP's are free of a lipoprotein envelope or a lipoprotein-containing envelope. In a further embodiment, the VLP's are free of an envelope altogether.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains. Methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the presently disclosed methods and compositions. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

**Equivalents and Scope**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments, described herein. The scope of the present disclosure is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The disclosure includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The disclosure includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the disclosure encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim. For

example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

Where elements are presented as lists, e.g., in Markush group format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should it be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not been specifically set forth in haec verba herein.

It is also noted that the term "comprising" is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term "comprising" is used herein, the term "consisting of" is thus also encompassed and disclosed

Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention (e.g., any nucleic acid or protein encoded thereby; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the

citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

## EXAMPLES

The disclosure will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the disclosure. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

### Example 1: Transfection of HuPBMCs with RLR Agonists Induces Cytokine Production *In Vitro*

To determine the effect of RLR agonists comprising various modifications on cytokine induction, the ability of RLR agonists to induce cytokine production was assessed *in vitro*. Human peripheral blood mononuclear cells (huPBMCs) were prepared from two healthy donors and seeded at a density of 2 X $10^5$ cells/well in a standard 96-well tissue culture plate in 100µL of RPMI 1640 cell culture medium supplemented with fetal calf serum (FCS), L-glutamine, and Pen/Strep. Independent transfections of huPBMCs with RLR agonists, as indicated in **FIG. 1**, were carried out using Lipofectamine2000 as the transfection reagent. Cells were incubated for 24 hours at $37^0$C in a humidified incubator followed by harvesting of cell culture supernatant. Supernatants were immediately frozen and stored at -$20^0$C. Samples were thawed once for analysis of cytokines IFN-α2a (**FIG. 1**), as well as IFN-β, IL-1β, IP-10, IL-12p70, IL-6, MCP-1 and MIP-1β (data not shown) using a U-Plex MSD platform according to the manufacturer's instructions. **FIG. 1** shows the dose-dependent induction of cytokine secretion from human PBMCs treated with novel candidate RLR agonists comprising various modifications and/or sequence motifs. RLR agonists were added at either 10nM, 2nM, or 0.4nM. The amount of cytokines released by the cells in response to RLR agonist transfection is given in pg/mL.

Tables 3 and 4 show the sequences of each RLR agonist. Table 3 also shows the sequence and number corresponding to each compound tested in **FIG. 1**. For example, compound X25224 in **FIG. 1** corresponds to "RIG7" which comprises a first oligonucleotide comprising SEQ ID NO: 42 linked via a linker "UUCG" to a second oligonucleotide comprising SEQ ID NO: 73 and has a

5’ diphosphate moiety. The sequence of RIG7 is also set forth as SEQ ID NO: 6 in Table 4. For RLR agonists in Table 3 having a first and second oligonucleotide connected via a nucleotide linker, the agonist is synthesized as a single oligonucleotide using an oligonucleotide synthesizer. As shown in Table 3, certain RLR agonists have a first oligonucleotide linked via a synthetic linker to a second oligonucleotide. For example, “RIG 43a” comprises a first oligonucleotide comprising SEQ ID NO: 63 linked via a C9 alkyl linker to a second oligonucleotide comprising SEQ ID NO: 91; “RIG 43b” comprises a first oligonucleotide comprising SEQ ID NO: 63 linked via a C9 alkyl linker to a second oligonucleotide comprising SEQ ID NO: 91 and a 5’ diphosphate moiety; and “RIG 44” comprises a first oligonucleotide comprising SEQ ID NO: 63 linked via a hexaethylene glycol linker to a second oligonucleotide comprising SEQ ID NO: 91 and a 5’ diphosphate moiety.

In a separate experiment, the potency of two RLR agonists in inducing IFN-α expression at different concentration was further evaluated. RIG 50c (X24907) (Linehan et al., (2018) Sci. Adv. 4(2):e1701854) and the inosine-substituted RIG 27c (X24935) were tested in the in vitro assay as described above at concentrations of 0.2 nM, 2 nM, 20 nM, and 200 nM. **FIG. 2** shows the dose-dependent induction of IFN-α expression by both compounds compared to cytokine levels induced by non-specific controls, i.e., PBMCs incubated with medium, lipofectamine, or immune serum. At the lower concentrations tested, incubating PBMCs with RIG 27c resulted in higher secretion of IFN-α compared to IFN-α levels elicited by incubation with RIG 50c. PBMCs transfected with 0.2 nM or 2 nM RIG 27c compared to PBMCs transfected with RIG 50c at the same concentrations elicited IFN-α levels of 498±87 pg/mL vs. 167.2±21 pg/mL and 3152±200 pg/mL vs. 2469±91 pg/mL, respectively. **FIG. 2**.

**TABLE 3: OLIGONUCLEOTIDE COMBINATION TABLE**

| RNA | First Oligonucleotide (FO) | Second Oligonucleotide (SO) | Linker | 5’ Phosphate Moiety |
|---|---|---|---|---|
| RIG 2a (X32671) | 37 | 68 | UUCG | - |
| RIG 2b (X25217) | 37 | 68 | UUCG | pp |
| RIG 3a (X32666) | 38 | 69 | UUCG | - |
| RIG 3b (X25218) | 38 | 69 | UUCG | pp |
| RIG 4 (X25219) | 39 | 70 | UUCG | pp |
| RIG 5 (X25221) | 40 | 71 | UUCG | pp |
| RIG 6 (X25222) | 41 | 72 | UUCG | pp |

| | | | | |
|---|---|---|---|---|
| RIG 7 (X25224) | 42 | 73 | UUCG | pp |
| RIG 8 (X25225) | 43 | 74 | UUCG | pp |
| RIG 9 (X25226) | 44 | 75 | UUCG | pp |
| RIG 10 (X25227) | 45 | 76 | UUCG | pp |
| RIG 11 (X25228) | 46 | 77 | UUCG | pp |
| RIG 12 (X25229) | 47 | 78 | UUCG | pp |
| RIG 13a (X32667) | 48 | 79 | UUCG | - |
| RIG 13b (X25230) | 48 | 79 | UUCG | pp |
| RIG 13c (X24921) | 48 | 79 | UUCG | ppp |
| RIG 14 (X25231) | 49 | 80 | UUCG | pp |
| RIG 15a (X32665) | 50 | 81 | UUCG | - |
| RIG 15b (X25232) | 50 | 81 | UUCG | pp |
| RIG 15c (X24923) | 50 | 81 | UUCG | ppp |
| RIG 16 (X25233) | 51 | 82 | UUCG | pp |
| RIG 18 (X25234) | 52 | 83 | UUCG | pp |
| RIG 20a (X32750) | 53 | 84 | UUCG | - |
| RIG 20b (X25235) | 53 | 84 | UUCG | pp |
| RIG 21 (X25237) | 54 | 85 | UUCG | pp |
| RIG 22a (X32672) | 55 | 86 | UUCG | - |
| RIG 22b (X25239) | 55 | 86 | UUCG | pp |
| RIG 24a (X25241) | 56 | 87 | UUCG | pp |
| RIG 24b (X25240) | 56 | 87 | UUCG | ppp |
| RIG 25 (X25242) | 57 | 88 | UUCG | pp |
| RIG 26 (X25243) | 58 | 89 | UUCG | pp |
| RIG 27a (X32669) | 59 | 89 | UUCG | - |
| RIG 27b (X25244) | 59 | 89 | UUCG | pp |
| RIG 27c (X24935) | 59 | 89 | UUCG | ppp |
| RIG 28a (X25245) | 60 | 90 | UUCG | pp |
| RIG 28b (X24936) | 60 | 90 | UUCG | ppp |
| RIG 35a (X32670) | 61 | 91 | UUCG | - |
| RIG 35b (X25247) | 61 | 91 | UUCG | pp |
| RIG 36 (X24945) | 62 | 92 | UUCG | pp |
| RIG 37a (X25249) | 63 | 91 | UUUGAU | pp |
| RIG 37b (X25248) | 63 | 91 | UUUGAU | ppp |
| RIG 38a (X32668) | 63 | 91 | UGUUU | - |
| RIG 38b (X25251) | 63 | 91 | UGUUU | pp |
| RIG 39 (X25253) | 63 | 91 | GAUC | pp |
| RIG 40 (X25255) | 64 | 93 | GAUC | pp |
| RIG 41 (X25257) | 65 | 94 | GAUC | pp |
| RIG 42 (X25259) | 64 | 93 | UUCG | pp |
| RIG 43a (X32673) | 63 | 91 | (C9) | - |
| RIG 43b (X25261) | 63 | 91 | (C9) | pp |
| RIG 44 (X25263) | 63 | 91 | (HEG) | pp |
| RIG 47 (X25265) | 66 | 95 | UUCG | pp |

| | | | | |
|---|---|---|---|---|
| RIG 48 (X25267) | 67 | 96 | UUCG | pp |
| RIG 49a (X25269) | 63 | 97 | UUCG | pp |
| RIG 49b (X25268) | 63 | 97 | UUCG | ppp |
| RIG 50a (14L) (X32664) | 63 | 91 | UUCG | - |
| RIG 50b (14L) (X24943) | 63 | 91 | UUCG | pp |
| RIG 50c (14L) (X24907) | 63 | 91 | UUCG | ppp |

(-) indicates no 5' phosphate; (pp) indicates 5' diphosphate; (ppp) indicates 5' triphosphate; HEG = hexaethylene glycol linker

**Example 2: Qβ VLP Synthesis**

Synthesis of Qβ VLP is described in U.S. Patent No. 9,518,095, incorporated herein by reference and described briefly below.

***Cloning Strategy for the Expression Plasmid pTac-nSD-Qb-Mut (SEQ ID NO:101)***

The coat protein-encoding gene (C) of E. coli RNA bacteriophage Qβ was amplified from plasmid pSDQb-rout (SEQ ID NO:109). The plasmid contains the sequence of gene C coding for the 133-aa Qβ coat protein (CP) and the 329-aa read through protein (A1). To prevent read-through, nucleotides 445-450 according to NCBI GenBank Acc. No. M99030 TGAACA (SEQ ID NO:102) are replaced by the sequence TAATGA (SEQ ID NO:103).

The coat protein-encoding gene C from plasmid pSDQb-mut was amplified by PCR. Oligonucleotide Qb-FOR3/2 (SEQ ID NO:104) with an internal EcoRI site and a synthetic Shine-Dalgarno (SD, SEQ ID NO:105) sequence anneals to the 5' end of the Qb CP gene. Oligonucleotide Qblang-REV2/2 (SEQ ID NO:106) contains an internal HindIII site and primes to the 3' end of the noncoding region of gene C. The 1054 bp amplified PCR fragment includes nucleotides 46-1062 of NCBI GenBank Acc. No. M99039 (except the nucleotide changes described above) and the synthetic SD sequence. The PCR fragment was digested with the restriction enzymes HindIII/EcoRI and the resulting 1036 bp fragment was inserted into the HindIII/EcoRI restriction sites of a modified pKK223-3 vector (Pharmacia, NCBI GenBank Acc. No.: M77749, SEQ ID NO:107). In this modified pKK223-3 vector the ampicillin resistance gene was replaced with the

kanamycin resistance gene of vector pUC4K (Pharmacia, NCBI GenBank Acc. No.: X06404, SEQ ID NO:108).

Vector pTac-nSDQb-mut (SEQ ID NO:109) differs from vector pTacQb-mut in the Shine-Dalgarno sequence. This Shine-Dalgarno sequence (nSD, SEQ ID NO:110) was introduced by amplifying the Qβ coat protein-encoding gene C via PCR from plasmid pTacQb-mut. Oligonucleotide nSDQb-mutEcoRIfor (SEQ ID NO:111) with an internal EcoRI site and the corresponding synthetic Shine-Dalgarno (nSD) sequence anneals to the 5' end of the Qb CP gene.

***Expression of Qβ CP Under Control of the Tac Promoter and nSD***

The E. coli strain RB791 was transformed with plasmids pTac-nSD-Qb-mut (SEQ ID NO:101). The clone was grown in shake flasks. Each flask contained 100 ml of R40 medium (main culture medium, Hypep 7455, glycerol) with kanamycin (25 μg/ml) and was inoculated with overnight cultures at a start $OD_{600}$ of 0.3. The shake flasks were incubated for 4 hours ($OD_{600}$ between 4 and 5) at 30ºC and an agitation of 220 rpm. The induction was carried out with 0.5% of lactose for 4 hours. Protein production was determined by SDS-PAGE. The gel showed a strong protein band which was identified as Qβ CP.

A scale-up process for manufacturing and purification of Qβ-VLP is disclosed in U.S. Patent No. 9,657,065, incorporated herein by reference. Briefly, E. coli cell pellets were suspended in a 0.1% (v/v) Triton-X-100 solution and disrupted by three passages at 700 ± 50 bar through an APV LAB 100 high pressure liquid homogenizer (HPLH). The homogenate was next processed by tangential flow filtration (TFF) and sterile filtration, and/or clarified by centrifugation.

The cell homogenate was further purified using Anion Exchange (AIX chromatography) on a FRACTOGEL.RTM.EMD TMAE column, followed by 0.22μM pore sterilizing filtration, and then loaded on a MACRO-PREP.RTM ceramic hydroxyapatite Type II column.

**Example 3: Packaging RLR agonists into VLP**

RLR agonists may be packaged into VLPs as described in U.S. Patent No. 9,950,055, incorporated herein by reference. Briefly, purified Qβ dimer protein is isolated as described in Example 2 above. The purified Qβ dimers capsid proteins are reassembled into VLP around the RLR agonists during the packaging step described below.

RLR agonist annealing is performed by dissolving an RLR agonist stock solution (3.3 mg/mL) in NaCP1 buffer (250 mM NaCI, 20 nM NaPi pH 7.2) to a concentration of 2.2 mg/mL. The mixture is heated to 70°C for 5 minutes, and cooled at room temperature for 15 minutes before placing on ice until Qβ reduction is completed. The Qβ dimer (4.165 mg/mL) is reduced in 5mM DTT and NaCP1 buffer. The Qβ dimer and RLR agonist are mixed by shaking at room temperature for 1 hour. $H_2O_2$ is then added to 10 mM, and the Qβ RLR agonist solution is shaken for another hour at room temperature. The Qβ RLR agonist reassembly mixture is then dialyzed with a 100 kDa MWCO membrane.

Alternatively, RLR agonists were dissolved in water and added to Qβ VLPs at 1, 10 and 100 nmol/ml in 0.2X HBS and incubated for 3 hours at 37° C in a thermomixer. Excess nucleic acids were removed by enzymatic hydrolysis or dialysis.

**Example 4: Transfection of HuPBMCs with Qβ RIG Induces Cytokine Production *In Vitro***

The ability of a Qβ-RIG made according to Example 3 above, Qβ-RIG27c (a RLR agonist with nucleotide sequence of SEQ ID NO:23 packaged into a VLP with RNA-phage Qβ coat protein having the amino acid sequence of SEQ ID NO: 112), to induce IFN-α expression at different concentration was further evaluated. Specifically, Qβ-RIG27c was tested in the in vitro assay as described above at concentrations of 2 nM, 20 nM, 200 nM, and 600 nM (concentration based on the RLR agonist). **FIG. 3** shows the dose-dependent induction of IFN-α expression by Qβ-RIG27c compared to cytokine expression levels elicited by non-specific controls, i.e., PBMCs incubated with medium, lipofectamine, or immune serum.

This result shows that the Qβ VLP delivery vehicle can be used to package RLR agonists and the resultant RIG-VLPs, e.g., Qβ-RIG27c, induce IFN-α expression from PBMCs in the presence of immune serum. While PBMCs transfected with an empty capsid, "capsid+serum" does not induce an IFN-α response.

**Example 5: In vivo efficacy of Qβ-RIG**

The in vivo efficacy of Qβ-RIGs may be evaluated in pre-clinical animal studies as described in U.S. Patent No. 9,950,055, incorporated herein by reference. Mice are subcutaneously administered with Qβ-RIGs. The immunostimulatory effect of Qβ-RIGs is

measured by intracellular cytokine staining of immune cells at various time-points post-administration.

In a prophylactic study, mice are subcutaneously primed with Qβ-RIGs and VLPs comprising a viral antigen (antigen-VLPs). At various time points post-prime, the frequency of antigen-specific are measured by tetramer staining. Mice are then challenged with live virus expressing the viral antigen, and the frequency of antigen-specific T cells are measured post-challenged in e.g., a tetramer assay. The immunostimulatory effect of Qβ-RIGS may be determined by comparing the frequency of antigen-specific T cells in mice that were primed with Qβ-RIGS and antigen-VLPs compared to mice primed with antigen-VLPs alone.

In a therapeutic study, mice are infected with live virus expressing a viral antigen. Tetramer staining is performed to measure the frequency of antigen-specific T cells post-infection. The mice are then subcutaneously administered Qβ-RIGS and antigen-VLPs, and the frequency of antigen-specific T cells are measured at various time-points post-administration. The immunostimulatory effect of Qβ-RIGS may be determined by comparing the frequency of antigen-specific T cells in mice that were administered Qβ-RIGS and antigen-VLPs compared to mice administered antigen-VLPs alone.

To evaluate the potency of Qβ-RIGs as anti-cancer therapy, mice are given bilateral subcutaneous administration of either A20 B cell lymphoma or B16F10 melanoma. Qβ-RIGs, or saline control, are delivered intratumorally (i.t.) on one flank starting 3-7 days after tumor challenge for a total of three doses to assess the local (treated tumor) and systemic (untreated tumor) effect of Qβ-RIG therapy. Body weights, tumor volumes (treated and untreated), and overall survival are measured.

The combination of Qβ-RIGs with an anti-cancer therapy, e.g., anti-PD1, is studied using the same tumor models. Mice are subcutaneously administered either A20 or B16F10 tumor cells and treated with Qβ-RIGs alone as described above, or in combination with anti-PD-1 or saline control delivered intraperitoneally twice weekly starting 3-7 days after tumor challenge. The study also includes an additional treatment arm with anti-PD-1 alone. Enhancement of anti-cancer efficacy is measured by comparing body weights, tumor volumes (treated and untreated), and overall survival of mice treated with anti-PD1 or Qβ-RIGs alone, or in combination.

## TABLE 4: SEQUENCE LISTING

| SEQ ID NO | Description | Sequence |
|---|---|---|
| 1 | RIG2 Nucleic acid sequence | GGATCGATCGATCGUUCGCGATCGATCGATCC |
| 2 | RIG3 Nucleic acid sequence | GGAUCGAUCGAUAUUUCGAUAUCGAUCGAUCC |
| 3 | RIG4 Nucleic acid sequence | GCGCGCGCGCGCGCUUCGGCGCGCGCGCGCGC |
| 4 | RIG5 Nucleic acid sequence | GGCGGCGCGCCGCCUUCGGGCGGCGCGCCGCC |
| 5 | RIG6 Nucleic acid sequence | GGCGGCGGCGGCGGUUCGCCGCCGCCGCCGCC |
| 6 | RIG7 Nucleic acid sequence | GGCGGCCGCCCGCGUUCGCGCGGGCGGCCGCC |
| 7 | RIG8 Nucleic acid sequence | CGACGUCGACGUCGUUCGCGACGUCGACGUCG |
| 8 | RIG9 Nucleic acid sequence | GCACGUCGACGUGCUUCGGCACGUCGACGUGC |
| 9 | RIG10 Nucleic acid sequence | GGACGUCGACGUCCUUCGGGACGUCGACGUCC |
| 10 | RIG11 Nucleic acid sequence | GGUCGCGACCAUAUUUCGAUAUGGUCGCGACC |
| 11 | RIG12 Nucleic acid sequence | GGAUACGUCGACGUUUCGACGUCGACGUAUCC |
| 12 | RIG13 Nucleic acid sequence | GAGAGAGAGAGAGAUUCGUCUCUCUCUCUCUC |
| 13 | RIG14 Nucleic acid sequence | GAGUCUAGACUCCGUUCGCGGAGUCUAGACUC |
| 14 | RIG15 Nucleic acid | CGAUCGAUCGAUCGUUCGCGAUCGAUCGAUCG |

| | | |
|---|---|---|
| | sequence (RIG 45) | |
| 15 | RIG16 Nucleic acid sequence | CCAUCGAUCGAUCGUUCGCGAUCGAUCGAUGG |
| 16 | RIG18 Nucleic acid sequence | GAAUCGAUCGAUCGUUCGCGAUCGAUCGAUUC |
| 17 | RIG20 Nucleic acid sequence | GGGAUCGAUCGUUCGCGAUCGAUCCC |
| 18 | RIG21 Nucleic acid sequence | CCCCCGAUCGAUCGUUCGCGAUCGAUCGGGGG |
| 19 | RIG22 Nucleic acid sequence | GTGTGTGTGTGTGTUUCGACACACACACACAC |
| 20 | RIG24 Nucleic acid sequence | GTGTGTGGAUCGAUUUCGAUCGAUCCACACAC |
| 21 | RIG25 Nucleic acid sequence | GGAICGAICGAICGUUCGCGAICGAICGAICC |
| 22 | RIG26 Nucleic acid sequence | IIAUCIAUCIAUCIUUCGCIAUCIAUCIAUCC |
| 23 | RIG27 Nucleic acid sequence | GGAUCIAUCIAUCIUUCGCIAUCIAUCIAUCC |
| 24 | RIG28 Nucleic acid sequence | GGIUCGIUCGIUCGUUCGCGIUCGIUCGIUCC |
| 25 | RIG35 Nucleic acid sequence | IGAUCGAUCGAUCGUUCGCGAUCGAUCGAUCC |
| 26 | RIG36 Nucleic acid sequence | AUCGAUCGAUCGUUCGCGAUCGAUCGAU |
| 27 | RIG37 Nucleic acid sequence | GGAUCGAUCGAUCGUUUGAUCGAUCGAUCGAUCC |
| 28 | RIG38 Nucleic acid sequence | GGAUCGAUCGAUCGUGUUUCGAUCGAUCGAUCC |

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|---|---|---|
| 29 | RIG39 Nucleic acid sequence | GGAUCGAUCGAUCGGAUCCGAUCGAUCGAUCC |
| 30 | RIG40 Nucleic acid sequence | GGCAUGCGACCUCUGUUUGAUCAAACAGAGGUCGCAUGCC |
| 31 | RIG41 Nucleic acid sequence | GGCAUGCGACCUCUGAUCAGAGGUCGCAUGCC |
| 32 | RIG42 Nucleic acid sequence | GGCAUGCGACCUCUGUUUUUCGAAACAGAGGUCGCAUGCC |
| 33 | RIG47 Nucleic acid sequence | TGCUCGAUCGAUCGUUCGCGAUCGAUCGAGCA |
| 34 | RIG48 Nucleic acid sequence | TCGUCGAUCGAUCGUUCGCGAUCGAUCGACGA |
| 35 | RIG49 Nucleic acid sequence | GGAUCGAUCGAUCGUUCGTGAUCGAUCGAUGG |
| 36 | RIG 0 Nucleic acid sequence (14L) | GGAUCGAUCGAUCGUUCGCGAUCGAUCGAUCC |
| 37 | FO1 Nucleic acid sequence | GGATCGATCGATCG |
| 38 | FO2 Nucleic acid sequence | GGAUCGAUCGAUAU |
| 39 | FO3 Nucleic acid sequence | GCGCGCGCGCGCGC |
| 40 | FO4 Nucleic acid sequence | GGCGGCGCGCCGCC |
| 41 | FO5 Nucleic acid sequence | GGCGGCGGCGGCGG |
| 42 | FO6 Nucleic acid sequence | GGCGGCCGCCCGCG |

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|---|---|---|
| 43 | FO7 Nucleic acid sequence | CGACGUCGACGUCG |
| 44 | FO8 Nucleic acid sequence | GCACGUCGACGUGC |
| 45 | FO9 Nucleic acid sequence | GGACGUCGACGUCC |
| 46 | FO10 Nucleic acid sequence | GGUCGCGACCAUAU |
| 47 | FO11 Nucleic acid sequence | GGAUACGUCGACGU |
| 48 | FO12 Nucleic acid sequence | GAGAGAGAGAGAGA |
| 49 | FO13 Nucleic acid sequence | GAGUCUAGACUCCG |
| 50 | FO14 Nucleic acid sequence | CGAUCGAUCGAUCG |
| 51 | FO15 Nucleic acid sequence | CCAUCGAUCGAUCG |
| 52 | FO16 Nucleic acid sequence | GAAUCGAUCGAUCG |
| 53 | FO17 Nucleic acid sequence | GGGAUCGAUCG |
| 54 | FO18 Nucleic acid sequence | CCCCCGAUCGAUCG |
| 55 | FO19 Nucleic acid sequence | GTGTGTGTGTGTGT |
| 56 | FO20 Nucleic acid sequence | GTGTGTGGAUCGAU |
| 57 | FO21 Nucleic acid sequence | GGAICGAICGAICG |

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|---|---|---|
| 58 | FO22 Nucleic acid sequence | IIAUCIAUCIAUCI |
| 59 | FO23 Nucleic acid sequence | GGAUCIAUCIAUCI |
| 60 | FO24 Nucleic acid sequence | GGIUCGIUCGIUCG |
| 61 | FO25 Nucleic acid sequence | IGAUCGAUCGAUCG |
| 62 | FO26 Nucleic acid sequence | AUCGAUCGAUCG |
| 63 | FO27 Nucleic acid sequence | GGAUCGAUCGAUCG |
| 64 | FO28 Nucleic acid sequence | GGCAUGCGACCUCUGUUU |
| 65 | FO29 Nucleic acid sequence | GGCAUGCGACCUCU |
| 66 | FO30 Nucleic acid sequence | TGCUCGAUCGAUCG |
| 67 | FO31 Nucleic acid sequence | TCGUCGAUCGAUCG |
| 68 | SO1 Nucleic acid sequence | CGATCGATCGATCC |
| 69 | SO2 Nucleic acid sequence | AUAUCGAUCGAUCC |
| 70 | SO3 Nucleic acid sequence | GCGCGCGCGCGCGC |
| 71 | SO4 Nucleic acid sequence | GGCGGCGCGCCGCC |
| 72 | SO5 Nucleic acid sequence | CCGCCGCCGCCGCC |

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|---|---|---|
| 73 | SO6 Nucleic acid sequence | CGCGGGCGGCCGCC |
| 74 | SO7 Nucleic acid sequence | CGACGUCGACGUCG |
| 75 | SO8 Nucleic acid sequence | GCACGUCGACGUGC |
| 76 | SO9 Nucleic acid sequence | GGACGUCGACGUCC |
| 77 | SO10 Nucleic acid sequence | AUAUGGUCGCGACC |
| 78 | SO11 Nucleic acid sequence | ACGUCGACGUAUCC |
| 79 | SO12 Nucleic acid sequence | UCUCUCUCUCUCUC |
| 80 | SO13 Nucleic acid sequence | CGGAGUCUAGACUC |
| 81 | SO14 Nucleic acid sequence | CGAUCGAUCGAUCG |
| 82 | SO15 Nucleic acid sequence | CGAUCGAUCGAUGG |
| 83 | SO16 Nucleic acid sequence | CGAUCGAUCGAUUC |
| 84 | SO17 Nucleic acid sequence | CGAUCGAUCCC |
| 85 | SO18 Nucleic acid sequence | CGAUCGAUCGGGGG |
| 86 | SO19 Nucleic acid sequence | ACACACACACACAC |
| 87 | SO20 Nucleic acid sequence | AUCGAUCCACACAC |

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|---|---|---|
| 88 | SO21 Nucleic acid sequence | CGAICGAICGAICC |
| 89 | SO22 Nucleic acid sequence | CIAUCIAUCIAUCC |
| 90 | SO23 Nucleic acid sequence | CGIUCGIUCGIUCC |
| 91 | SO24 Nucleic acid sequence | CGAUCGAUCGAUCC |
| 92 | SO25 Nucleic acid sequence | CGAUCGAUCGAU |
| 93 | SO26 Nucleic acid sequence | AAACAGAGGUCGCAUGCC |
| 94 | SO27 Nucleic acid sequence | AGAGGUCGCAUGCC |
| 95 | SO28 Nucleic acid sequence | CGAUCGAUCGAGCA |
| 96 | SO29 Nucleic acid sequence | CGAUCGAUCGACGA |
| 97 | SO30 Nucleic acid sequence | TGAUCGAUCGAUGG |
| 98 | Human RIG-I Amino acid sequence | MTTEQRRSLQAFQDYIRKTLDPTYILSYMAPWFREEEVQYIQAEKNN<br>KGPMEAATLFLKFLLELQEEGWFRGFLDALDHAGYSGLYEAIESWDF<br>KKIEKLEEYRLLLKRLQPEFKTRIIPTDIISDLSECLINQECEEILQ<br>ICSTKGMMAGAEKLVECLLRSDKENWPKTLKLALEKERNKFSELWIV<br>EKGIKDVETEDLEDKMETSDIQIFYQEDPECQNLSENSCPPSEVSDT<br>NLYSPFKPRNYQLELALPAMKGKNTIICAPTGCGKTFVSLLICEHHL<br>KKFPQGQKGKVVFFANQIPVYEQQKSVFSKYFERHGYRVTGISGATA<br>ENVPVEQIVENNDIIILTPQILVNNLKKGTIPSLSIFTLMIFDECHN<br>TSKQHPYNMIMFNYLDQKLGGSSGPLPQVIGLTASVGVGDAKNTDEA<br>LDYICKLCASLDASVIATVKHNLEELEQVVYKPQKFFRKVESRISDK<br>FKYIIAQLMRDTESLAKRICKDLENLSQIQNREFGTQKYEQWIVTVQ<br>KACMVFQMPDKDEESRICKALFLYTSHLRKYNDALIISEHARMKDAL<br>DYLKDFFSNVRAAGFDEIEQDLTQRFEEKLQELESVSRDPSNENPKL<br>EDLCFILQEEYHLNPETITILFVKTRALVDALKNWIEGNPKLSFLKP<br>GILTGRGKTNQNTGMTLPAQKCILDAFKASGDHNILIATSVADEGID<br>IAQCNLVILYEYVGNVIKMIQTRGRGRARGSKCFLLTSNAGVIEKEQ |

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|---|---|---|
| | | INMYKEKMMNDSILRLQTWDEAVFREKILHIQTHEKFIRDSQEKPKP<br>VPDKENKKLLCRKCKALACYTADVRVIEECHYTVLGDAFKECFVSRP<br>HPKPKQFSSFEKRAKIFCARQNCSHDWGIHVKYKTFEIPVIKIESFV<br>VEDIATGVQTLYSKWKDFHFEKIPFDPAEMSK |
| 99 | Human MDA5 Amino acid sequence | MSNGYSTDENFRYLISCFRARVKMYIQVEPVLDYLTFLPAEVKEQIQ<br>RTVATSGNMQAVELLLSTLEKGVWHLGWTREFVEALRRTGSPLAARY<br>MNPELTDLPSPSFENAHDEYLQLLNLLQPTLVDKLLVRDVLDKCMEE<br>ELLTIEDRNRIAAAENNGNESGVRELLKRIVQKENWFSAFLNVLRQT<br>GNNELVQELTGSDCSESNAEIENLSQVDGPQVEEQLLSTTVQPNLEK<br>EVWGMENNSSESSFADSSVVSESDTSLAEGSVSCLDESLGHNSNMGS<br>DSGTMGSDSDEENVAARASPEPELQLRPYQMEVAQPALEGKNIIICL<br>PTGSGKTRVAVYIAKDHLDKKKKASEPGKVIVLVNKVLLVEQLFRKE<br>FQPFLKKWYRVIGLSGDTQLKISFPEVVKSCDIIISTAQILENSLLN<br>LENGEDAGVQLSDFSLIIIDECHHTNKEAVYNNIMRHYLMQKLKNNR<br>LKKENKPVIPLPQILGLTASPGVGGATKQAKAEEHILKLCANLDAFT<br>IKTVKENLDQLKNQIQEPCKKFAIADATREDPFKEKLLEIMTRIQTY<br>CQMSPMSDFGTQPYEQWAIQMEKKAAKEGNRKERVCAEHLRKYNEAL<br>QINDTIRMIDAYTHLETFYNEEKDKKFAVIEDDSDEGGDDEYCDGDE<br>DEDDLKKPLKLDETDRFLMTLFFENNKMLKRLAENPEYENEKLTKLR<br>NTIMEQYTRTEESARGIIFTKTRQSAYALSQWITENEKFAEVGVKAH<br>HLIGAGHSSEFKPMTQNEQKEVISKFRTGKINLLIATTVAEEGLDIK<br>ECNIVIRYGLVTNEIAMVQARGRARADESTYVLVAHSGSGVIEHETV<br>NDFREKMMYKAIHCVQNMKPEEYAHKILELQMQSIMEKKMKTKRNIA<br>KHYKNNPSLITFLCKNCSVLACSGEDIHVIEKMHHVNMTPEFKELYI<br>VRENKALQKKCADYQINGEIICKCGQAWGTMMVHKGLDLPCLKIRNF<br>VVVFKNNSTKKQYKKWVELPITFPNLDYSECCLFSDED |
| 100 | Human LGP2 Amino acid sequence | MELRSYQWEVIMPALEGKNIIIWLPTGAGKTRAAAYVAKRHLETVDG<br>AKVVVLVNRVHLVTQHGEEFRRMLDGRWTVTTLSGDMGPRAGFGHLA<br>RCHDLLICTAELLQMALTSPEEEEHVELTVFSLIVVDECHHTHKDTV<br>YNVIMSQYLELKLQRAQPLPQVLGLTASPGTGGASKLDGAINHVLQL<br>CANLDTWCIMSPQNCCPQLQEHSQQPCKQYNLCHRRSQDPFGDLLKK<br>LMDQIHDHLEMPELSRKFGTQMYEQQVVKLSEAAALAGLQEQRVYAL<br>HLRRYNDALLIHDTVRAVDALAALQDFYHREHVTKTQILCAERRLLA<br>LFDDRKNELAHLATHGPENPKLEMLEKILQRQFSSSNSPRGIIFTRT<br>RQSAHSLLLWLQQQQGLQTVDIRAQLLIGAGNSSQSTHMTQRDQQEV<br>IQKFQDGTLNLLVATSVAEEGLDIPHCNVVVRYGLLTNEISMVQARG<br>RARADQSVYAFVATEGSRELKRELINEALETLMEQAVAAVQKMDQAE<br>YQAKIRDLQQAALTKRAAQAAQRENQRQQFPVEHVQLLCINCMVAVG<br>HGSDLRKVEGTHHVNVNPNFSNYYNVSRDPVVINKVFKDWKPGGVIS<br>CRNCGEVWGLQMIYKSVKLPVLKVRSMLLETPQGRIQAKKWSRVPFS<br>VPDFDFLQHCAENLSDLSLD |
| 101 | pTac-nSD-Qb-Mut plasmid | GGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCG<br>CACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTC<br>TGGCAAATATTCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGT<br>ATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG<br>AATTCTAAGGAGGAAAAAAAAATGGCAAAATTAGAGACTGTTACTTT<br>AGGTAACATCGGGAAAGATGGAAAACAAACTCTGGTCCTCAATCCGC |

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| | | GTGGGGTAAATCCCACTAACGGCGTTGCCTCGCTTTCACAAGCGGGT<br>GCAGTTCCTGCGCTGGAGAAGCGTGTTACCGTTTCGGTATCTCAGCC<br>TTCTCGCAATCGTAAGAACTACAAGGTCCAGGTTAAGATCCAGAACC<br>CGACCGCTTGCACTGCAAACGGTTCTTGTGACCCATCCGTTACTCGC<br>CAGGCATATGCTGACGTGACCTTTTCGTTCACGCAGTATAGTACCGA<br>TGAGGAACGAGCTTTTGTTCGTACAGAGCTTGCTGCTCTGCTCGCTA<br>GTCCTCTGCTGATCGATGCTATTGATCAGCTGAACCCAGCGTATTAA<br>TGACTGCTCATTGCCGGTGGTGGCTCAGGGTCAAAACCCGATCCGGT<br>TATTCCGGATCCACCGATTGATCCGCCGCCAGGGACAGGTAAGTATA<br>CCTGTCCCTTCGCAATTTGGTCCCTAGAGGAGGTTTACGAGCCTCCT<br>ACTAAGAACCGACCGTGGCCTATCTATAATGCTGTTGAACTCCAGCC<br>TCGCGAATTTGATGTTGCCCTCAAAGATCTTTTGGGCAATACAAAGT<br>GGCGTGATTGGGATTCTCGGCTTAGTTATACCACGTTCCGCGGTTGC<br>CGTGGCAATGGTTATATTGACCTTGATGCGACTTATCTTGCTACTGA<br>TCAGGCTATGCGTGATCAGAAGTATGATATTCGCGAGGGCAAGAAAC<br>CTGGTGCTTTCGGTAACATTGAGCGATTCATTTATCTTAAGTCGATA<br>AATGCTTATTGCTCTCTTAGCGATATTGCGGCCTATCACGCCGATGG<br>CGTGATAGTTGGCTTTTGGCGCGATCCATCCAGTGGTGGTGCCATAC<br>CGTTTGACTTCACTAAGTTTGATAAGACTAAATGTCCTATTCAAGCC<br>GTGATAGTCGTTCCTCGTGCTTAGTAACTAAGGATGAAATGCATGTC<br>TAAGCTTGGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATAC<br>AGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTTGCCT<br>GGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGA<br>AGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGA<br>GAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAA<br>AGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCC<br>TGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAA<br>CGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCA<br>TCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTC<br>TACAAACTCTTTTGTTTATTTTTCTAGAGCCACGTTGTGTCTCAAAA<br>TCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAA<br>TAAAACTGTCTGCTTACATAAACAGTAATACAAGGAGTGTTATGAGC<br>CATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAA<br>CATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCG<br>GGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCGATGCG<br>CCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGT<br>TACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTC<br>TTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTA<br>CTCACCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAAGA<br>ATATCCTGATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCC<br>TGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGC<br>GATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGG<br>TTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTG<br>TTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGCCATTCTCACCG<br>GATTCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTT<br>TGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAA<br>TCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGT |

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| | | GAGTTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTAT<br>TGATAATCCTGATATGAATAAATTGCAGTTTCATTTGATGCTCGATG<br>AGTTTTTCTAAACGCGTGACCAAGTTTACTCATATGTACTTTAGATT<br>GATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCT<br>TTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCC<br>ACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGAT<br>CCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACC<br>GCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTT<br>TTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTC<br>CTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGC<br>ACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTG<br>CCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAG<br>TTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCAC<br>ACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTAC<br>AGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCG<br>GACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAG<br>GGAGCTCCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGT<br>TTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGG<br>GGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTT<br>CCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTAT<br>CCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGAT<br>ACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGA<br>GGAAGCGGAAGAGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGT<br>GCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCT<br>GATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGAC<br>TGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCC<br>CTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGA<br>CCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCG<br>AAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAG<br>CGATTCACAGATGTCTGCCTGTTCATCCGCGTCCAGCTCGTTGAGTT<br>TCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTA<br>AGGGCGGTTTTTTCCTGTTTGGTCACTGATGCCTCCGTGTAAGGGGG<br>ATTTCTGTTCATGGGGGTAATGATACCGATGAAACGAGAGAGGATGC<br>TCACGATACGGGTTACTGATGATGAACATGCCCGGTTACTGGAACGT<br>TGTGAGGGTAAACAACTGGCGGTATGGATGCGGCGGGACCAGAGAAA<br>AATCACTCAGGGTCAATGCCAGCGCTTCGTTAATACAGATGTAGGTG<br>TTCCACAGGGTAGCCAGCAGCATCCTGCGATGCAGATCCGGAACATA<br>ATGGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAACACG<br>GAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGTTTTGC<br>AGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCTGC<br>TAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAG<br>GAGCACGATCATGCGCACCCGTGGCCAGGACCCAACGCTGCCCGAGA<br>TGCGCCGCGTGCGGCTGCTGGAGATGGCGGACGCGATGGATATGTTC<br>TGCCAAGGGTTGGTTTGCGCATTCACAGTTCTCCGCAAGAATTGATT<br>GGCTCCAATTCTTGGAGTGGTGAATCCGTTAGCGAGGTGCCGCCGGC<br>TTCCATTCAGGTCGAGGTGGCCCGGCTCCATGCACCGCGACGCAACG<br>CGGGGAGGCAGACAAGGTATAGGGCGGCGCCTACAATCCATGCCAAC |

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| | | CCGTTCCATGTGCTCGCCGAGGCGGCATAAATCGCCGTGACGATCAG<br>CGGTCCAATGATCGAAGTTAGGCTGGTAAGAGCCGCGAGCGATCCTT<br>GAAGCTGTCCCTGATGGTCGTCATCTACCTGCCTGGACAGCATGGCC<br>TGCAACGCGGGCATCCCGATGCCGCCGGAAGCGAGAAGAATCATAAT<br>GGGGAAGGCCATCCAGCCTCGCGTCGCGAACGCCAGCAAGACGTAGC<br>CCAGCGCGTCGGCCGCCATGCCGGCGATAATGGCCTGCTTCTCGCCG<br>AAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAGCGAGGGCGTG<br>CAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTCGCGCTCC<br>AGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGGCACC<br>TGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGAC<br>GATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGG<br>CTCTCAAGGGCATCGGTCGACGCTCTCCCTTATGCGACTCCTGCATT<br>AGGAAGCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGC<br>AAGGAATGGTGCATGCAAGGAGATGGCGCCCAACAGTCCCCCGGCCA<br>CGGGGCCTGCCACCATACCCACGCCGAAACAAGCGCTCATGAGCCCG<br>AAGTGGCGAGCCCGATCTTCCCCATCGGTGATGTCGGCGATATAGGC<br>GCCAGCAACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGTC<br>CGGCGTAGAGGATCCGGGCTTATCGACTGCACGGTGCACCAATGCTT<br>CTGGCGTCAGGCAGCCATCGGAAGCTGTGGTAT |
| 102 | Stop codon | TGAACA |
| 103 | Stop codon | TAATGA |
| 104 | Qb-FOR3/2 sequence | GCGCGCGAATTCAGGAGGTAAAAAACGATGGCAAAATTAGAGACTGT<br>TACTTTAGG |
| 105 | Shine-Dalgano sequence | AGGAGGTAAAAAACGATG |
| 106 | Qblang-REV2/2 sequence | GCATGCAAGCTTAGACATGCATTTCATCCTTAG |
| 107 | modified pKK223-3 cloning vector | TTCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACATT<br>ATACGAGCCGATGATTAATTGTCAACAGCTCATTTCAGAATATTTGC<br>CAGAACCGTTATGATGTCGGCGCAAAAAACATTATCCAGAACGGGAG<br>TGCGCCTTGAGCGACACGAATTATGCAGTGATTTACGACCTGCACAG<br>CCATACCACAGCTTCCGATGGCTGCCTGACGCCAGAAGCATTGGTGC<br>ACCGTGCAGTCGATAAGCTCCGGATCCTCTACGCCGGACGCATCGTG<br>GCCGGCATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGC<br>CGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTCATGA<br>GCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGA<br>CTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGT<br>GCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGT<br>CGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCA<br>GTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACT<br>TATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAG<br>CGCTCTGGGTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGCGACG<br>ATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACGCCCTCGC<br>TCAAGCCTTCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGC |

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| | | AGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTTG<br>CTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCCATTATGATTCT<br>TCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCATGCTGT<br>CCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTC<br>GCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTCAC<br>GGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGA<br>TTGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGC<br>GGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCAC<br>CTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTT<br>GCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAACATATCCA<br>TCGCGTCCGCCATCTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGC<br>GTTGGGTCCTGGCCACGGGTGCGCATGATCGTGCTCCTGTCGTTGAG<br>GACCCGGCTAGGCTGGCGGGGTTGCCTTACTGGTTAGCAGAATGAAT<br>CACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCT<br>GCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTA<br>AAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGA<br>TCTGCATCGCAGGATGCTGCTGGCTACCCTGTGGAACACCTACATCT<br>GTATTAACGAAGCGCTGGCATTGACCCTGAGTGATTTTTCTCTGGTC<br>CCGCCGCATCCATACCGCCAGTTGTTTACCCTCACAACGTTCCAGTA<br>ACCGGGCATGTTCATCATCAGTAACCCGTATCGTGAGCATCCTCTCT<br>CGTTTCATCGGTATCATTACCCCCATGAACAGAAATTCCCCCTTACA<br>CGGAGGCATCAAGTGACCAAACAGGAAAAAACCGCCCTTAACATGGC<br>CCGCTTTATCAGAAGCCAGACATTAACGCTTCTGGAGAAACTCAACG<br>AGCTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGAC<br>CACGCTGATGAGCTTTACCGCAGCTGCCTCGCGCGTTTCGGTGATGA<br>CGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTT<br>GTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCA<br>GCGGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAG<br>CGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGAT<br>TGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCG<br>TAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCTTCCTCGCTCACT<br>GACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA<br>CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAG<br>GAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAA<br>AAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA<br>GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAG<br>GACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGC<br>TCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCT<br>CCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATC<br>TCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA<br>CCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCT<br>TGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCA<br>CTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG<br>TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATT<br>TGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTG<br>GTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT<br>TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGA |

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| | | AGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAA<br>ACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC<br>ACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAG<br>TATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTG<br>AGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCC<br>TGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATC<br>TGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTC<br>CAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA<br>AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTG<br>CCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACG<br>TTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGT<br>ATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATG<br>ATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGA<br>TCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATG<br>GCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTT<br>TTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTA<br>TGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACC<br>GCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTC<br>TTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT<br>CGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACT<br>TTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC<br>AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCT<br>TCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG<br>AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAAGAGTTT<br>GTAGAAACGCAAAAAGGCCATCCGTCAGGATGGCCTTCTGCTTAATT<br>TGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGG<br>CCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTAC<br>TCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAG<br>TCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTA<br>CTCTCGCATGGGGAGACCCCACACTACCATCGGCGCTACGGCGTTTC<br>ACTTCTGAGTTCGGCATGGGGTCAGGTGGGACCACCGCGCTACTGCC<br>GCCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATTTAA<br>TCTGTATCAGGCTGAAAATCTTCTCTCATCCGCCAAAACAGAAGCTT<br>GGCTGCAGGTCGACGGATCCCCGGGAA |
| 108 | modified pKK223-3 cloning vector | TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTC<br>CCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACA<br>AGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGC<br>TTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATG<br>CGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAG<br>GCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGG<br>TGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCT<br>GCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACG<br>TTGTAAAACGACGGCCAGTGAATTCCCCGGATCCGTCGACCTGCAGG<br>GGGGGGGGGGCGCTGAGGTCTGCCTCGTGAAGAAGGTGTTGCTGACT<br>CATACCAGGCCTGAATCGCCCCATCATCCAGCCAGAAAGTGAGGGAG<br>CCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGATTTT<br>GAACTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCG |

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| | | TGATCTGATCCTTCAACTCAGCAAAAGTTCGATTTATTCAACAAAGC<br>CGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTTACAACCAA<br>TTAACCAATTCTGATTAGAAAAACTCATCGAGCATCAAATGAAACTG<br>CAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCC<br>GTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATG<br>GCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAAT<br>ACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGA<br>AATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTA<br>TGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTC<br>ATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCG<br>CCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAA<br>ACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAAC<br>AATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTG<br>TTTTCCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTA<br>CGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCA<br>GTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTT<br>TGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAAT<br>CGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTT<br>ATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCG<br>AGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTA<br>CTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTT<br>ATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTT<br>CCCCCCCCCCCCCTGCAGGTCGACGGATCCGGGGAATTCGTAATCATG<br>GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC<br>ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAA<br>TGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTT<br>CCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC<br>GCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCG<br>CTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATC<br>AGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATA<br>ACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAAC<br>CGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCC<br>TGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACC<br>CGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC<br>GTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGC<br>CTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTA<br>GGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTG<br>CACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTA<br>TCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAG<br>CAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCT<br>ACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGAC<br>AGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA<br>GAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT<br>GGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATC<br>TCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGA<br>ACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGG<br>ATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT |

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| | | CTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAA<br>TCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATA<br>GTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTT<br>ACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCAC<br>CGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAG<br>CGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAA<br>TTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGC<br>GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCG<br>TTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGT<br>TACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTC<br>CTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATG<br>GTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAG<br>ATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAAT<br>AGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGAT<br>AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAA<br>ACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT<br>CCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCT<br>TTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAA<br>TGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCA<br>TACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGT<br>CTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT<br>AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAG<br>AAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACG<br>AGGCCCTTTCGTC |
| 109 | pSDQb-rout plasmid | TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTC<br>CCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACA<br>AGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGC<br>TTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATG<br>CGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAG<br>GCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGG<br>TGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCT<br>GCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACG<br>TTGTAAAACGACGGCCAGTGAATTCAGACATGCATTTCATCCTTAGT<br>TACTAAGCACGAGGAACGACTATCACGGCTTGAATAGGACATTTAGT<br>CTTATCAAACTTAGTGAAGTCAAACGGTATGGCACCACCACTGGATG<br>GATCGCGCCAAAAGCCAACTATCACGCCATCGGCGTGATAGGCCGCA<br>ATATCGCTAAGAGAGCAATAAGCATTTATCGACTTAAGATAAATGAA<br>TCGCTCAATGTTACCGAAAGCACCAGGTTTCTTGCCCTCGCGAATAT<br>CATACTTCTGATCACGCATAGCCTGATCAGTAGCAAGATAAGTCGCA<br>TCAAGGTCAATATAACCATTGCCACGGCAACCGCGGAACGTGGTATA<br>ACTAAGCCGAGAATCCCAATCACGCCACTTTGTATTGCCCAAAAGAT<br>CTTTGAGGGCAACATCAAATTCGCGAGGCTGGAGTTCAACAGCATTA<br>TAGATAGGCCACGGTCGGTTCTTAGTAGGAGGCTCGTAAACCTCCTC<br>TAGGGACCAAATTGCGAAGGGACAGGTATACTTACCTGTCCCTGGCG<br>GCGGATCAATCGGTGGATCCGGAATAACCGGATCGGGTTTTGACCCT<br>GAGCCACCACCGGCAATGAGCAGTCATTAATACGCTGGGTTCAGCTG<br>ATCAATAGCATCGATCAGCAGAGGACTAGCGAGCAGAGCAGCAAGCT |

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| | | CTGTACGAACAAAAGCTCGTTCCTCATCGGTACTATACTGCGTGAAC<br>GAAAAGGTCACGTCAGCATATGCCTGGCGAGTAACGGATGGGTCACA<br>AGAACCGTTTGCAGTGCAAGCGGTCGGGTTCTGGATCTTAACCTGGA<br>CCTTGTAGTTCTTACGATTGCGAGAAGGCTGAGATACCGAAACGGTA<br>ACACGCTTCTCCAGCGCAGGAACTGCACCCGCTTGTGAAAGCGAGGC<br>AACGCCGTTAGTGGGATTTACCCCACGCGGATTGAGGACCAGAGTTT<br>GTTTTCCATCTTTCCCGATGTTACCTAAAGTAACAGTCTCTAATTTT<br>GCCATCGTTTTTTACCTCCTTCTAGAGTCATTATGGTTTTGCCATAC<br>ATCAGTATGGTGTAGCAGCACTTATTATAATCTTTATTGCCTCTTAA<br>AACTTAATCCACATCAAAACTCAAATACTTTTAACCCCAGCGTCCTG<br>TAAGCTCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTT<br>GCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT<br>CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTA<br>ATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTG<br>AGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC<br>TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAAT<br>CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA<br>CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGA<br>CCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGC<br>GTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTA<br>GGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGC<br>CCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCG<br>GTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGAT<br>TAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGT<br>GGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCT<br>CTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATC<br>CGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGC<br>AGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATC<br>TTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGG<br>GATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTT<br>TAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAA<br>ACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTC<br>AGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCG<br>TGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT<br>GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGC<br>AATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAA<br>CTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGA<br>GTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGC<br>TACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCA<br>GCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTG<br>TGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAG<br>TAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATA<br>ATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGT<br>GAGTGGGGGGGGGGGGCGCTGAGGTCTGCCTCGTGAAGAAGGTGTTG<br>CTGACTCATACCAGGCCTGAATCGCCCCATCATCCAGCCAGAAAGTG<br>AGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGT<br>GATTTTGAACTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAA |

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| | | GATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTCGATTTATTCAA<br>CAAAGCCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTTAC<br>AACCAATTAACCAATTCTGATTAGAAAAACTCATCGAGCATCAAATG<br>AAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAA<br>AAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCAT<br>AGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAAC<br>ATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAA<br>GTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAA<br>AGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACG<br>CTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTG<br>ATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAA<br>TTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGC<br>ATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGA<br>ATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCA<br>GGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGT<br>CAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGC<br>TACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCA<br>TACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGC<br>CCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCG<br>GCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTT<br>GTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATAT<br>ATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGT<br>GGCTTTCCCCCCCCCCCCATTATTGAAGCATTTATCAGGGTTATTGT<br>CTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT<br>AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAG<br>AAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACG<br>AGGCCCTTTCGTC |
| 110 | Shine-Dalgano sequence | TAAGGAGGAAAAAAAAATG |
| 111 | nSDQb-mutEcoRIfor sequence | GCGCGCGAATTCTAAGGAGGAAAAAAAAATGGCAAAATTAGAGACTG<br>TTACTTTAGG |
| 112 | RNA-phage Qβ CP | MAKLETVTLGNIGKDGKQTLVLNPRGVNPTNGVASLSQAGAVPALEK<br>RVTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQAYADVT<br>FSFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY |
| 113 | RNA-phage Qβ A1 protein | MAKLETVTLGNIGKDGKQTLVLNPRGVNPTNGVASLSQAGAVPALEK<br>RVTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQAYADVT<br>FSFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAYWTLLIAGG<br>GSGSKPDPVIPDPPIDPPPGTGKYTCPFAIWSLEEVYEPPTKNRPWP<br>IYNAVELQPREFDVALKDLLGNTKWRDWDSRLSYTTFRGCRGNGYID<br>LDATYLATDQAMRDQKYDIREGKKPGAFGNIERFIYLKSINAYCSLS<br>DIAAYHADGVIVGFWRDPSSGGAIPFDFTKFDKTKCPIQAVIVVPRA |
| 114 | bacteriophage R17 | ASNFTQFVLVNDGGTGNVTVAPSNFANGVAEWISSNSRSQAYKVTCS<br>VRQSSAQNRKYTIKVEVPKVATQTVGGVELPVAAWRSYLNMELTIPI<br>FATNSDCELIVKAMQGLLKDGNPIPSAIAANSGIY |

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| 115 | bacteriophage fr | MASNFEEFVLVDNGGTGDVKVAPSNFANGVAEWISSNSRSQAYKVTC<br>SVRQSSANNRKYTVKVEVPKVATQVQGGVELPVAAWRSYMNMELTIP<br>VFATNDDCALIVKALQGTFKTGNPIATAIAANSGIY |
| 116 | bacteriophage GA | MATLRSFVLVDNGGTGNVTVVPVSNANGVAEWLSNNSRSQAYRVTAS<br>YRASGADKRKYAIKLEVPKIVTQVVNGVELPGSAWKAYASIDLTIPI<br>FAATDDVTVISKSLAGLFKVGNPIAEAISSQSGFYA |
| 117 | bacteriophage SP CP | MAKLNQVTLSKIGKNGDQTLTLTPRGVNPTNGVASLSEAGAVPALEK<br>RVTVSVAQPSRNRKNFKVQIKLQNPTACTRDACDPSVTRSAFADVTL<br>SFTSYSTDEERALIRTELAALLADPLIVDAIDNLNPAY |
| 118 | bacteriophage SP A1 protein | AKLNQVTLSKIGKNGDQTLTLTPRGVNPTNGVASLSEAGAVPALEKR<br>VTVSVAQPSRNRKNFKVQIKLQNPTACTRDACDPSVTRSAFADVTLS<br>FTSYSTDEERALIRTELAALLADPLIVDAIDNLNPAYWAALLVASSG<br>GGDNPSDPDVPVVPDVKPPDGTGRYKCPFACYRLGSIYEVGKEGSPD<br>IYERGDEVSVTFDYALEDFLGNTNWRNWDQRLSDYDIANRRRCRGNG<br>YIDLDATAMQSDDFVLSGRYGVRKVKFPGAFGSIKYLLNIQGDAWLD<br>LSEVTAYRSYGMVIGFWTDSKSPQLPTDFTQFNSANCPVQTVIIIPS |
| 119 | bacteriophage MS2 | MASNFTQFVLVDNGGTGDVTVAPSNFANGVAEWISSNSRSQAYKVTC<br>SVRQSSAQNRKYTIKVEVPKVATQTVGGVELPVAAWRSYLNMELTIP<br>IFATNSDCELIVKAMQGLLKDGNPIPSAIAANSGIY |
| 120 | bacteriophage M11 | MAKLQAITLSGIGKKGDVTLDLNPRGVNPTNGVAALSEAGAVPALEK<br>RVTISVSQPSRNRKNYKVQVKIQNPTSCTASGTCDPSVTRSAYSDVT<br>FSFTQYSTVEERALVRTELQALLADPMLVNAIDNLNPAY |
| 121 | bacteriophage MX1 | MAKLQAITLSGIGKNGDVTLNLNPRGVNPTNGVAALSEAGAVPALEK<br>RVTISVSQPSRNRKNYKVQVKIQNPTSCTASGTCDPSVTRSAYADVT<br>FSFTQYSTDEERALVRTELKALLADPMLIDAIDNLNPAY |
| 122 | bacteriophage NL95 | MAKLNKVTLTGIGKAGNQTLTLTPRGVNPTNGVASLSEAGAVPALEK<br>RVTVSVAQPSRNRKNYKVQIKLQNPTACTKDACDPSVTRSGSRDVTL<br>SFTSYSTERERALIRTELAALLKDDLIVDAIDNLNPAYWAALLAASP<br>GGGNNPYPGVPDSPNVKPPGGTGTYRCPFACYRRGELITEAKDGACA<br>LYACGSEALVEFEYALEDFLGNEFWRNWDGRLSKYDIETHRRCRGNG<br>YVDLDASVMQSDEYVLSGAYDVVKMQPPGTFDSPRYYLHLMDGIYVD<br>LAEVTAYRSYGMVIGFWTDSKSPQLPTDFTRFNRHNCPVQTVIVIPS<br>L |
| 123 | bacteriophage f2 | ASNFTQFVLVNDGGTGNVTVAPSNFANGVAEWISSNSRSQAYKVTCS<br>VRQSSAQNRKYTIKVEVPKVATQTVGGVELPVAAWRSYLNLELTIPI<br>FATNSDCELIVKAMQGLLKDGNPIPSAIAANSGIY |
| 124 | bacteriophage PP7 | MSKTIVLSVGEATRTLTEIQSTADRQIFEEKVGPLVGRLRLTASLRQ<br>NGAKTAYRVNLKLDQADVVDCSTSVCGELPKVRYTQVWSHDVTIVAN<br>STEASRKSLYDLTKSLVATSQVEDLVVNLVPLGR |
| 125 | Qβ–240 (K13R) | AKLETVTLGNIG**R**DGKQTLVLNPRGVNPTNGVASLSQAGAVPALEKR<br>VTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQKYADVTF<br>SFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY |
| 126 | Qβ-243 (N10K) | AKLETVTLG**K**IGKDGKQTLVLNPRGVNPTNGVASLSQAGAVPALEKR<br>VTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQKYADVTF<br>SFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY |

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| 127 | Qβ-250 (K2R, K13R) | A**R**LETVTLGNIG**R**DGKQTLVLNPRGVNPTNGVASLSQAGAVPALEKR VTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQKYADVTF SFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY |
| 128 | Qβ -251 | AKLETVTLGNIGKDGRQTLVLNPRGVNPTNGVASLSQAGAVPALEKR VTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQKYADVTF SFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY |
| 129 | Qβ-259 (K2R, K16R) | A**R**LETVTLGNIGKDG**R**QTLVLNPRGVNPTNGVASLSQAGAVPALEKR VTVSVSQPSRNRKNYKVQVKIQNPTACTANGSCDPSVTRQKYADVTF SFTQYSTDEERAFVRTELAALLASPLLIDAIDQLNPAY |
| 130 | Plasmid pAP283-58 | CGAGCTCGCCCCTGGCTTATCGAAATTAATACGACTCACTATAGGGA GACCGGAATTCGAGCTCGCCCGGGGATCCTCTAGAATTTTCTGCGCA CCCATCCCGGGTGGCGCCCAAAGTGAGGAAAATCACATGGCAAATAA GCCAATGCAACCGATCACATCTACAGCAAATAAAATTGTGTGGTCGG ATCCAACTCGTTTATCAACTACATTTTCAGCAAGTCTGTTACGCCAA CGTGTTAAAGTTGGTATAGCCGAACTGAATAATGTTTCAGGTCAATA TGTATCTGTTTATAAGCGTCCTGCACCTAAACCGGAAGGTTGTGCAG ATGCCTGTGTCATTATGCCGAATGAAAACCAATCCATTCGCACAGTG ATTTCAGGGTCAGCCGAAAACTTGGCTACCTTAAAAGCAGAATGGGA AACTCACAAACGTAACGTTGACACACTCTTCGCGAGCGGCAACGCCG GTTTGGGTTTCCTTGACCCTACTGCGGCTATCGTATCGTCTGATACT ACTGCTTAAGCTTGTATTCTATAGTGTCACCTAAATCGTATGTGTAT GATACATAAGGTTATGTATTAATTGTAGCCGCGTTCTAACGACAATA TGTACAAGCCTAATTGTGTAGCATCTGGCTTACTGAAGCAGACCCTA TCATCTCTCTCGTAAACTGCCGTCAGAGTCGGTTTGGTTGGACGAAC CTTCTGAGTTTCTGGTAACGCCGTTCCGCACCCCGGAAATGGTCACC GAACCAATCAGCAGGGTCATCGCTAGCCAGATCCTCTACGCCGGACG CATCGTGGCCGGCATCACCGGCGCACACAGTGCGGTTGCTGGCGCCT ATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGG CTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGC CGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGG CGGCGGTGCTTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAAT GCAGGAGTCGCATAAGGGAGAGCGTCGATATGGTGCACTCTCAGTAC AATCTGCTCTGATGCCGCATAGTTAAGCCAACTCCGCTATCGCTACG TGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACG CGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCT GTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATC ACCGAAACGCGCGAGGCAGCTTGAAGACGAAAGGGCCTCGTGATACG CCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGT CAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTA TTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACC CTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTC AACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTT CCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGA AGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACA GCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATG ATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTAT |

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| | | TGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGA<br>ATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGAT<br>GGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGA<br>TAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGG<br>AGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTT<br>GATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCG<br>TGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTAT<br>TAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGAC<br>TGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCT<br>TCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTG<br>GGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCC<br>CGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGA<br>ACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATT<br>GGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTA<br>AAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGA<br>TAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAG<br>CGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT<br>TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACC<br>AGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGA<br>AGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTA<br>GTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCC<br>TACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTG<br>GCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCG<br>GATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCC<br>CAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGCG<br>AGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGG<br>TATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCT<br>TCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCC<br>ACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGG<br>AGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC<br>CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTG<br>ATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCT<br>CGCCGCAGCCGAACGACGAGCGCAGCGAGTCAGTGAGCGAGGAAGCG<br>GAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGAT<br>TCATTAATGCAGCTGTGGTGTCATGGTCGGTGATCGCCAGGGTGCCG<br>ACGCGCATCTCGACTGCATGGTGCACCAATGCTTCTGGCGTCAGGCA<br>GCCATCGGAAGCTGTGGTATGGCCGTGCAGGTCGTAAATCACTGCAT<br>AATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTG<br>CGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGCTGTTG<br>ACAATTAATCATCGAACTAGTTAACTAGTACGCAAGTTCACGTAAAA<br>AGGGTATCGCGGAATT |
| 131 | AP205 | tctagaATTTTCTGCGCACCCATCCCGGGTGGCGCCCAAAGTGAGGA<br>AAATCACatg |
| 132 | Shine delgarno sequence | tctagaTTAACCCAACGCGTAGGAGTCAGGCCatg |

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| 133 | AP205 coat protein | MANKPMQPITSTANKIVWSDPTRLSTTFSASLLRQRVKVGIAELNNV<br>SGQYVSVYKRPAPKPEGCADACVIMPNENQSIRTVISGSAENLATLK<br>AEWETHKRNVDTLFASGNAGLGFLDPTAAIVSSDTTA |
| 134 | AP205 coat protein (P5T) | MANK**T**MQPITSTANKIVWSDPTRLSTTFSASLLRQRVKVGIAELNNV<br>SGQYVSVYKRPAPKPEGCADACVIMPNENQSIRTVISGSAENLATLK<br>AEWETHKRNVDTLFASGNAGLGFLDPTAAIVSSDTTA |
| 135 | Plasmid pAP281-32 | CGAGCTCGCCCCTGGCTTATCGAAATTAATACGACTCACTATAGGGA<br>GACCGGAATTCGAGCTCGCCCGGGGATCCTCTAGATTAACCCAACGC<br>GTAGGAGTCAGGCCATGGCAAATAAGACAATGCAACCGATCACATCT<br>ACAGCAAATAAAATTGTGTGGTCGGATCCAACTCGTTTATCAACTAC<br>ATTTTCAGCAAGTCTGTTACGCCAACGTGTTAAAGTTGGTATAGCCG<br>AACTGAATAATGTTTCAGGTCAATATGTATCTGTTTATAAGCGTCCT<br>GCACCTAAACCGAAGGTCAGATGCCTGTGTCATTATGCCGAATGAAA<br>ACCAATCCATTCGCACAGTGATTTCAGGGTCAGCCGAAAACTTGGCT<br>ACCTTAAAAGCAGAATGGGAAACTCACAAACGTAACGTTGACACACT<br>CTTCGCGAGCGGCAACGCCGGTTTGGGTTTCCTTGACCCTACTGCGG<br>CTATCGTATCGTCTGATACTACTGCTTAAGCTTGTATTCTATAGTGT<br>CACCTAAATCGTATGTGTATGATACATAAGGTTATGTATTAATGGTA<br>GCCGCGTTCTAACGACAATATGTACAAGCCTAATTGTGTAGCATCTG<br>GCTTACTGAAGCAGACCCTATCATCTCTCTCGTAAACTGCCGTCAGA<br>GTCGGTTGGGTTGGACAGACCTCTGAGTTTCTGGTAACGCCGTTCCG<br>CACCCCGGAAATGGTCACCGAACCATTCAGCAGGGTCATCGCTAGCC<br>AGATCCTCTACGCCGGACGCATCGTGGCCCGCATCACCGGCGCCACA<br>GGTGCGGTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGAT<br>CGGGCTCGCCACTTCGGGCTCATGATCGCTGGTTTCCGCCTGGGTAT<br>GGTGGCAGGCCCCGTGGCCCGGGGGACTGTTGGGCGCCATCTCCTTG<br>CATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACT<br>ACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGAT<br>ATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCC<br>AACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACC<br>CGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTTCCGGCA<br>TCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCA<br>GAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTTGAAGACG<br>AAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA<br>TAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGC<br>GGACCCCCTATTGGTTTATTTTTCTAAATACATTCAAATATGTATCC<br>GCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAA<br>GGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTT<br>TTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGT<br>GAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACA<br>TCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCC<br>GAAGAACGTTTTTCAATGATGAGCACTTTTAAAGTTCTGCTATGTGT<br>CGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCC<br>GCATACACTATTCTCAGAATGACTTGGTGGTACCTACCAGTCACAGA<br>AAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTG<br>CCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACG<br>ATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGA |

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| | | TCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCA<br>TACCAAACGACGAGCGTGACACCACGATGCCTGTACGAACGGCAACA<br>ACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCG<br>GCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCAC<br>TTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCT<br>GGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCC<br>AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTC<br>AGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCC<br>TCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATAT<br>ACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGG<br>TGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAG<br>TTTTCGTTCCACTGAGCGGTCAGACCCCGTAGAAAGATCAAAGGATC<br>TTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAA<br>AAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTA<br>CCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACC<br>AAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGA<br>ACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCA<br>GTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTC<br>AAGACGATAGGTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGG<br>GTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTG<br>AGATACCTACAGCGCGAGCATTGAGAAAGCGCCACGCTTCCCGAAGG<br>GAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAAGAG<br>AGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGT<br>CCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATG<br>CTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCT<br>TTTTACGGTTCCTGGCCTTTGGCTGGCCTTTTGCTCACATGTTCTTT<br>CCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGA<br>GTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGACGGCGCAGCGA<br>GTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTC<br>TCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGTGGTGTCATGGTC<br>GGTGATCGCCAGGGTGCCGACGCGCATCTCGACTGCATGGTGCACCA<br>ATGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCCGTGC<br>AGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCG<br>TTCTGGATAATGTTTTTTGCGGCGACATCATAACGGTTCTGGCAAAT<br>ATTCTGAAATGAGCTGGTGACAATTAATCATCGAACTAGTTAACTAG<br>TACGCAAGTTCACGTAAAAAGGGTATCGCGGAATT |
| 136 | HBcAG protein | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNYVNTNMG<br>LKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRDRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQCL |
| 137 | HBcAG protein variant | MDIDPYEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEHC<br>SPHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNYVNTNMGL<br>KIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILST<br>LPETTVVRRRDRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 138 | HBcAG protein | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGGNLEDPISRDLVVSYVNTNMG |

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| | variant | LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRGSQC |
| 139 | HBcAG protein variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGGNLEDPTSRDLVVSYVNTNMG<br>LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPTNAPILS<br>TLPETCVIRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRGSQC |
| 140 | HBcAG protein variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGGNLEDPISRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 141 | HBcAG protein variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDNASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGGNLEDPISRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 142 | HBcAG protein variant | MDIDPYKEFGATVELLSFLPTDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVSYVNTNMG<br>LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETCVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 143 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>MTLATWVGGNLEDPVSRDLVVSYVNTNVGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 144 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWDMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>MTLATWVGGNLEDPVSRDLVVSYVNTNVGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 145 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPQHTALRQAILCWGEL<br>MTLATWVGGNLEDPISRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 146 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYKPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRGSQC |
| 147 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALFRDALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGGNLEDPASRDLVVSYVNTNMG<br>LKFRQLLWFHISCLTFGRDTVIEYLVSFGVWIRTPPAYRPSNAPILS<br>TLPETCVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 148 | HBcAG protein | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVSYVNTNMG |

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| | Variant | LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 149 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRHAILCWGDL RTLATWVGGNLEDPISRDLVVSYVNTNMGLKFRQLLYFHISCLTFGR ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR TPSPRRRRSQSPRRRRSQSRESQC |
| 150 | HBcAG protein variant | MQLFHLCLIISCSCPTVQASKLCLGWLWDMDIDPYKEFGATVELLSF LPSDFFPSVRDLLDTASALFRDALESPEHCSPHHTALRQAILCWGEL MTLATWVGANLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR ETVIEYLVSFGVWIRTPQAYRPPNAPILSTLPETCVVRRRGRSPRRR TPSPRRRRSQSPRRRRSQSRESQC |
| 151 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH CSPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVSYVNTNMG LKFRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILS TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 152 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL MSLATWVGVNLEDPISRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR TPSPRRRRSQSPRRRRSQSRESQC |
| 153 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYRDALESPEH CSPHHTALRQAILCWGELMTLATWVGVNLEDPASRDLVVSYVNTNMG LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 154 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH CSPHHTALRQAILCWGDLMTLATWVGVNLEDPASRDLVVSYVNTNMG LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 155 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYRDALESPEH CSPHHTALRQAILCWGELMTLATWVGANLEDPASRDLVVSYVNTNMG LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS TLPETTVVRRRGRTPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 156 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF LPSDFFPSVRDLLDTASALYRDALESPEHCSPHHTALRQAILCWGEL MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR TPSPRRRRSQSPRRRRSQSRESQC |
| 157 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR TPSPRRRRSQSPRRRRSQSRESQC |
| 158 | HBcAG protein Variant | MQLFHLCLIISCTCPTVQASKLCLGWLWGMDIDPYKQFGATVELLSF LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGEL MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR |

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| | | ETVIEYLVAFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 159 | HBcAG protein variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREAFECSEHCSPHHTALRQAILCWGEL<br>MTLATWVGGNLEDPISRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 160 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLXAADMDIDPYKEFGATVELL<br>SFLPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWG<br>DLITLSTWVGGNLEDPTSRDLVVSYVNTNMGLKFRQLLWFHISCLTF<br>GRETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPR<br>RRTPSPRRRRSQSPRRRRTQSRESQC |
| 161 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDNASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 162 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHICCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 163 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSREPQC |
| 164 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLSTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 165 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILLTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 166 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFKQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 167 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTAAALYRDALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGTNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR |

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| | | ETVLEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 168 | HBcAG protein variant | MDIDPYKEFGASMELLSFLPSDFYPSVRDLLDTASALYREALESPEH<br>CTPHHTALRQAILCWGELMTLATWVGGNLQDPTSRDLVVSYVNTNMG<br>LKFRQLLWFHVSCLTFGRETVVEYLVSFGVWIRTPQAYRPPNAPILS<br>TLPETCVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 169 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRHVFLCWGDLMTLATWVGGNLEDPTSRDLVVSYVNTNMG<br>LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 170 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>TTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 171 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYRDALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLIFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 172 | HBcAG protein variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGDLMTLATWVGVNLEDPVSRDLVVSYVNTNVG<br>LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRGRSPRRRTPSPARRRSQSPRRRRSQSRESQC |
| 173 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>MNLATWVGGNLEDPVSRDLVVGYVNTTVGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPPRRRRSQSPRRRRSQSRESQC |
| 174 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYRDALESPEH<br>CSPHHTALRQAILCWGDLMTLATWVGVNLEDPASRDLVVSYVNTNMG<br>LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRGRTPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 175 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRALLDTASALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQILWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 176 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>MTLATWVGVNLEDPATRDLVVSYVNTNVGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 177 | HBcAG protein variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTASALYREALESPEHCSPHHTALRQRILCWGEL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR |

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|---|---|---|
| | | ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRTRSQSRESQC |
| 178 | HBcAG protein Variant | MQLFHLCLVISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSDFFPSVRDLLDTAAALYREALESPEHCSPHHTALRQAILCWGEL<br>MTLATWVGNNLEDPASRDLVVNYVNTNMGLKIRQLLWFHISCLTFGR<br>ETVLEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 179 | HBcAG protein Variant | MQLFHLCLIISCSCPTVQASKLCLGWLWGMDIDPYKEFGATVELLSF<br>LPSAFFPSVRDLLDTASALYREALESPEHCSPHHTALRQAILCWGDL<br>MTLATWVGVNLEDPASRDLVVSYVNTNMGLKFRQLLWFHISCLTFGR<br>ETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRGRSPRRR<br>TPSPRRRRSQSPRRRRSQSRESQC |
| 180 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAAALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNYVNTNMG<br>LKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |
| 181 | HBcAG protein Variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGGNLEDPISRDLVVSYVNTNMG<br>LKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETCVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRGSQC |
| 182 | HBcAG protein Variant | MDIDPYKEFGSSYQLLNFLPLDFFPDLNALVDTATALYEEELTGREH<br>CSPHHTAIRQALVCWDELTKLIAWMSSNITSEQVRTIIVNHVNDTWG<br>LKVRQSLWFHLSCLTFGQHTVQEFLVSFGVWIRTPAPYRPPNAPILS<br>TLPEHTVIRRRGGARASRSPRRRTPSPRRRRSQSPRRRRSQSPSTNC |
| 183 | HBcAG protein Variant | MYLFHLCLVFACVPCPTVQASKLCLGWLWDMDIDPYKEFGSSYQLLN<br>FLPLDFFPDLNALVDTAAALYEEELTGREHCSPHHTAIRQALVCWEE<br>LTRLITWMSENTTEEVRRIIVDHVNNTWGLKVRQTLWFHLSCLTFGQ<br>HTVQEFLVSFGVWIRTPAPYRPPNAPILSTLPEHTVIRRRGGSRAAR<br>SPRRRTPSPRRRRSQSPRRRRSQSPASNC |
| 184 | HBcAG protein Variant | MDVNASRALANVYDLPDDFFPKIEDLVRDAKDALEPYWKSDSIKKHV<br>LIATHFVDLIEDFWQTTQGMHEIAEAIRAVIPPTTAPVPSGYLIQHD<br>EAEEIPLGDLFKEQEERIVSFQPDYPITARIHAHLKAYAKINEESLD<br>RARRLLWWHYNCLLWGEATVTNYISRLRTWLSTPEKYRGRDAPTIEA<br>ITRPIQVAQGGRKTSTATRKPRGLEPRRRKVKTTVVYGRRRSKSRER<br>RASSPQRAGSPLPRSSSSHHRSPSPRK |
| 185 | HBcAG protein variant | MWDLRLHPSPFGAACQGIFTSSLLLFLVTVPLVCTIVYDSCLCMDIN<br>ASRALANVYDLPDDFFPKIDDLVRDAKDALEPYWRNDSIKKHVLIAT<br>HFVDLIEDFWQTTQGMHEIAEALRAIIPATTAPVPQGFLVQHEEAEE<br>IPLGELFRYQEERLTNFQPDYPVTARIHAHLKAYAKINEESLDRARR<br>LLWWHYNCLLWGEPNVTNYISRLRTWLSTPEKYRGKDAPTIEAITRP<br>IQVAQGGRNKTQGVRKSRGLEPRRRRVKTTIVYGRRRSKSRERRAPT<br>PQRAGSPLPRTSRDHHRSPSPRE |
| 186 | HBcAG protein variant | MDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALESPEH<br>CSPHHTALRQAILCWGELMTLATWVGNNLEDPASRDLVVNYVNTNMG<br>LKIRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILS<br>TLPETTVVRRRDRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC |

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|---|---|---|
| 187 | Amino acid sequence | GGKGG |
| 188 | Amino acid linker | (G)kC(G)m(S)l(GGGGS)n with n=0-3, k=0-5, m=0-10, l=0-2 |
| 189 | Amino acid linker | (G)m(S)l(GGGGS)n(G)oC(G)k with n=0-3, k=0-5, m=0-10, l=0-2, and o=0-8 |
| 190 | Amino acid linker | (GGGGS)n |
| 191 | Amino acid linker | CGDKTHTSPP |
| 192 | Amino acid linker | DKTHTSPPCG |
| 193 | Amino acid linker | CGGPKPSTPPGSSGGAP |
| 194 | Amino acid linker | PKPSTPPGSSGGAPGGCG |
| 195 | Amino acid linker | GCGGGG |
| 196 | Amino acid linker | GGGGCG |
| 197 | Amino acid linker | GGKKGC |
| 198 | Amino acid linker | CGKKGG |
| 199 | Amino acid linker | CGKKGG |
| 200 | Amino acid linker | CGDEGG |
| 201 | Amino acid linker | GGKKGC |
| 202 | Amino acid linker | GGEDGC |
| 203 | Amino acid linker | GGCG |

## CLAIMS

1. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RIG-I like receptor (RLR) agonist that specifically binds to a RLR, wherein the RLR agonist comprises a ribonucleic acid (RNA) of 10-100 nucleotides in length, wherein the 5' most nucleotide of the RNA comprises a 5'diphosphate or triphosphate moiety, or derivative or analog thereof,

wherein the at least one RLR agonist is packaged in the virus-like particle.

2. The composition of claim 1, wherein the RNA is single-stranded.

3. The composition of claim 1, wherein the RNA is double-stranded.

4. The composition of any one of claims 1-3, wherein the RNA is 10-15, 15-20, 20-25, 25-30 or 30-35 nucleotides in length.

5. The composition of claim 1, wherein the RLR agonist comprises a first polynucleotide and a second polynucleotide, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex.

6. The composition of claim 5, wherein the duplex comprises a hairpin.

7. The composition of any one of claims 5-6, wherein the duplex comprises 10-15, 15-20, 20-25, 25-30 or 30-35 base pairs.

8. The composition of any one of claims 5-6, wherein the duplex comprises less than 19 base pairs.

9. The composition of any one of claims 5-8, wherein the first polynucleotide is connected to the second polynucleotide by a linker.

10. The composition of any one of claims 5-9, wherein the RLR agonist comprises a sequence motif that provides at least one biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif.

11. The composition of claim 10, wherein the sequence motif is selected from the group consisting of:

(i) a GT-repeat motif;

(ii) a GA-repeat motif;

(iii) a AUCG-repeat motif;

(iv) an AU-repeat motif;

(v) a dipyrimidine motif;

(vi) a dipurine motif;

(vii) a pyrimidine triplet motif;

(viii) a purine triplet motif;

(ix) a palindromic sequence motif; and

(x) a combination of any of (i)-(ix).

12. The composition of any one of claims 10-11, wherein the at least one improved biological activity is selected from:

(i) an increase in RLR-mediated cytokine production;

(ii) an increase in RLR-mediated expression of interferon-stimulated genes;

(iii) an increase in RLR-mediated intracellular signaling;

(iv) an increase in binding affinity to RLRs; and

(v) a combination of any of (i)-(iv).

13. The composition of claims 10-12, wherein the sequence motif is a GT-repeat motif comprising a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 5-10, about 5, about 4, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and thymine nucleotides, or derivatives or analogs thereof.

14. The composition of claim 13, where in the GT-repeat motif is $[GT]_n$, wherein n = 2 to 9.

15. The composition of any one of claims 13-14, wherein the GT-repeat motif is $[GT]_7$.

16. The composition of any one of claims 13-14, wherein the GT-repeat motif is $[GT]_3$, and wherein the GT-repeat motif is followed by a purine triplet motif and UCG, respectively.

17. The composition of claim 16, wherein the purine triplet motif is GGA.

18. The composition of any one of claims 10-12, wherein the sequence motif is a GA-repeat motif comprising a sequence of <19, about 15-18, about 15, about 10-15, about 10, about 5-10, about 5, about 4, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 guanine and adenine nucleotides, or derivatives or analogs thereof.

19. The composition of claim 18, wherein the GA-repeat motif is $[GA]_n$, where n = 2 to 9.

20. The composition of claim 19, wherein the GA-repeat motif is $[GA]_7$.

21. The composition of any one of claims 10-12, wherein the sequence motif is a AUCG-repeat motif comprising a sequence of <19, about 16, about 12-16, about 12, about 8-12, about 6, 16, 12, 8 adenine, uracil, cytosine, and guanine nucleotides, or derivatives or analogs thereof.

22. The composition of claim 21, wherein the AUCG-repeat motif is $[AUCG]_n$, where n = 2 to 4.

23. The composition of claim 22, wherein the AUCG-repeat motif is $[AUCG]_3$.

24. The composition of any one of claims 21-23, wherein the AUCG-repeat motif is preceded by a CG or a dipyrimidine motif.

25. The composition of claim 24, wherein the AUCG-repeat motif is preceded by a CG.

26. The composition of claim 24, wherein the dipyrimidine motif is CC.

27. The composition of any one of claims 21-23, wherein the AUCG-repeat motif is preceded by a dipurine motif.

28. The composition of claim 27, wherein the dipurine motif is GA.

29. The composition of claim 27, wherein the dipurine motif is II.

30. The composition of any one of claims 21-29, wherein the U comprising the AUCG-repeat motif is substituted with a modified nucleoside.

31. The composition of claim 30, wherein the modified nucleoside is ribothymidine (T).

32. The composition of any one of claims 21-29, wherein the G comprising the AUCG-repeat motif is substituted with a modified nucleoside.

33. The composition of claim 32, wherein the modified nucleoside is inosine (I).

34. The composition of any one of claims 21-23, wherein the AUCG-repeat motif is preceded by an IG.

35. The composition of any one of claims 21-23, wherein the G comprising the AUCG-repeat is substituted with an inosine (I), and wherein the AUCG-repeat is preceded by an inosine (I).

36. The composition of claim 35, wherein the 5’ most nucleotide of the first polynucleotide is inosine (I).

37. The composition of any one of claims 21- 22, wherein the AUCG-repeat motif is $[AUCG]_2$.

38. The composition of claim 37, wherein the AUCG-repeat motif is preceded by a dipurine motif.

39. The composition of claim 38, wherein the dipurine motif is GG.

40. The composition of claim 37, wherein the AUCG-repeat motif is preceded by a purine triplet.

41. The composition of claim 40, wherein the purine triplet is GGG.

42. The composition of claim 37, wherein the AUCG-repeat motif is preceded by CCCCCG.

43. The composition of claim 37, wherein the AUCG-repeat motif is preceded by TCGUCG.

44. The composition of any one of claims 10-12, wherein the sequence motif is a palindromic sequence comprising a sequence of <19, about 15-18, about 15, about 10-15, about 10, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 nucleotides, or derivatives or analogs thereof, linked in any order that results in a palindrome.

45. The composition of any one of claims 9-44, wherein the linker is flanked by AU.

46. The composition of any one of claims 9-45, wherein the linker is flanked by an AU-repeat motif, wherein the AU-repeat motif is $[AU]_n$, where n = 2 to 3.

47. The composition of claim 46, wherein the AU-repeat motif is $[AU]_2$.

48. A composition comprising:

(a) a virus-like particle; and

(b) at least one RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is a linker that operably links the first polynucleotide and the second polynucleotide,

wherein at least one of N1, N2, N3, and N4 is inosine and/or at least one of X1 and/or X2 comprises at least one inosine nucleoside, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

49. The composition of claim 48, wherein N1 comprises inosine and N4 comprises cytidine.

50. The composition of claim 48, wherein N1 comprises cytidine and N4 comprises inosine.

51. The composition of claim 48, wherein N2 comprise inosine and N3 comprises cytidine.

52. The composition of claim 48, wherein N2 comprises cytidine and N3 comprises inosine.

53. The composition of claim 48, wherein N1 comprises guanosine.

54. The composition of claim 48, wherein N2 comprises guanosine.

55. The composition of claim 48, wherein N1 comprises cytidine.

56. The composition of claim 48, wherein N2 comprises cytidine.

57. The composition of claim 48, wherein N1 and N2 comprise guanosine and N3 and N4 comprise cytidine.

58. The composition of claim 48, wherein N1 and N2 comprise cytidine and N3 and N4 comprise guanosine.

59. The composition of claim 48, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine.

60. The composition of claim 48, wherein N1 and N2 comprise cytidine and N3 and N4 comprise inosine.

61. The composition of claim 48, wherein N1 comprises inosine and N4 comprises cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

62. The composition of claim 48, wherein N2 comprises inosine and N3 comprises cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

63. The composition of claim 48, wherein N1 and N2 comprise guanosine N3 and N4 comprise cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

64. The composition of claim 48, wherein N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and wherein X1 and X2 each comprise at least one inosine.

65. The composition of claim 48, wherein N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and wherein X1 and X2 each comprise at least one inosine.

66. The composition of claim 48, wherein N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and wherein X1 and X2 each comprise inosine and no guanosine nucleosides.

67. The composition of claim 48, wherein N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and wherein X1 and X2 each comprise inosine and no guanosine nucleosides.

68. The composition of claim 48, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

69. The composition of claim 48, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and wherein X1 and X2 each comprise at least one inosine.

70. The composition of claim 48, wherein N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and wherein X1 and/or X2 each comprise at least one inosine.

71. The composition of claim 48, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and wherein X1 and X2 comprise inosine and no guanosine nucleosides.

72. The composition of claim 48, wherein N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and wherein X1 and X2 comprise inosine and no guanosine nucleosides.

73. The composition of claim 48, wherein X1 and X2 are each 12 nucleotides and comprise 1, 2, 3 or 4 inosine nucleosides

74. The composition of claim 48, wherein X1 and X2 are each 13 nucleotides and comprise 1, 2, 3, 4 or 5 inosine nucleosides.

75. The composition of claim 48, wherein X1 and X2 are each 14 nucleotides and comprise 1, 2, 3, 4, 5 or 6 inosine nucleosides.

76. The composition of claim 48, wherein X1 and X2 are each 15 nucleotides and comprise 1, 2, 3, 4, 5, 6, or 7 inosine nucleosides

77. The composition of claim 48, wherein X1 and X2 are each 16 nucleotides and each comprise 1, 2, 3, 4, 5, 6, 7, or 8 inosine nucleosides.

78. The composition of claim 48, wherein X1 and X2 are each 12 nucleotides and comprise at least 10%, 20%, 30% or 40% inosine nucleosides.

79. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ comprises a sequence motif $[AUCN_5]_x$ , wherein $N_5$ comprises guanosine or inosine, wherein x is an integer whose value indicates the number of sequence motifs, and wherein x= 2-4;

(viii) $X_2$ comprises a sequence motif $[CN_6AU]_y$, wherein N6 comprises guanosine or inosine, wherein y is an integer whose value indicates the number of sequence motifs, and wherein y = 2-4;

(ix) L is a linker that operably links the first polynucleotide and the second polynucleotide,

optionally, wherein at least one of N1, N2, N3, and N4 is inosine, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

80. The composition of claim 79, wherein N5 comprises inosine and N6 comprises inosine.

81. The composition of claim 80, wherein N5 comprises guanosine and N6 comprises inosine.

82. The composition of claim 81, wherein N5 comprises inosine and N6 comprises guanosine.

83. The composition of claim 82, wherein N5 comprises guanosine (G) and N6 comprises guanosine (G).

84. The composition of any one of claims 79-83, wherein (i) x=2 and y=2 or (ii) x = 3 and y = 3.

85. The composition of any one of claims 79-83, wherein x = 4 and y = 4.

86. The composition of any one of claim 79-83, wherein N1 comprises inosine (I) and N4 comprises cytidine (C).

87. The composition of any one of claims 79-83, wherein N2 comprises inosine (I) and N3 comprises cytidine (C).

88. The composition of any one of claims 79-83, wherein N3 comprises inosine (I) and N2 comprises cytidine (C).

89. The composition of any one of claims 79-83, wherein N4 comprises inosine (I) and N1 comprises cytidine (C).

90. The composition of any one of claims 79-83, wherein N1 comprises guanosine (G)

91. The composition of any one of claims 79-83, wherein N2 comprises guanosine (G).

92. The composition of any one of claims 79-83, wherein N1 comprises cytidine (C).

93. The composition of any one of claims 79-83, wherein N2 comprises cytidine (C).

94. The composition of any one of claims 79-83, wherein N1 and N2 comprise guanosine (G) and N3 and N4 comprise cytidine (C).

95. The composition of any one of claims 79-83, wherein N1 and N2 comprise cytidine (C) and N3 and N4 comprise guanosine (G).

96. The composition of any one of claims 79-83, wherein N1 and N2 comprise inosine (I) and N3 and N4 comprise cytidine (C).

97. The composition of any one of claims 79-83, wherein N1 and N2 comprise cytidine (C) and N3 and N4 comprise inosine (I).

98. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, and wherein the agonist comprises the formula:

5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein

(i) ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide comprising linked nucleotides $N_1$, $N_2$ and $X_1$;

(ii) ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide comprising linked nucleotides $X_2$, $N_3$ and $N_4$;

(iii) $N_1$, $N_2$, $N_3$ and $N_4$ each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) $N_1$ base pairs with $N_4$;

(v) $N_2$ base pairs with $N_3$;

(vi) $N_1$ comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) $X_1$ and $X_2$ are each oligonucleotides comprising nucleosides selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(viii) $X_1$ is complementary to $X_2$;

(ix) $X_1$ and $X_2$ are each 12 nucleotides to 16 nucleotides in length and are the same length, and;

(x) L is the non-nucleotide linker that covalently links the first polynucleotide and the second polynucleotide,

wherein inosine, if present, base pairs with cytidine, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

99. The composition of claim 98, wherein N1 comprises inosine and N4 comprises cytidine.

100. The composition of claim 98, wherein N1 comprises cytidine and N4 comprises inosine.

101. The composition of claim 98, wherein N2 comprise inosine and N3 comprises cytidine.

102. The composition of claim 98, wherein N2 comprises cytidine and N3 comprises inosine.

103. The composition of claim 98, wherein N1 comprises guanosine.

104. The composition of claim 98, wherein N2 comprises guanosine.

105. The composition of claim 98, wherein N1 comprises cytidine.

106. The composition of claim 98, wherein N2 comprises cytidine.

107. The composition of claim 98, wherein N1 and N2 comprise guanosine and N3 and N4 comprise cytidine.

108. The composition of claim 98, wherein N1 and N2 comprise cytidine and N3 and N4 comprise guanosine.

109. The composition of claim 98, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine.

110. The composition of claim 98, wherein N1 and N2 comprise cytidine and N3 and N4 comprise inosine.

111. The composition of claim 98, wherein N1 comprises inosine and N4 comprises cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

112. The composition of claim 98, wherein N2 comprises inosine and N3 comprises cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

113. The composition of claim 98, wherein N1 and N2 comprise guanosine N3 and N4 comprise cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

114. The composition of claim 98, wherein N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and wherein X1 and X2 each comprise at least one inosine.

115. The composition of claim 98, wherein N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and wherein X1 and X2 each comprise at least one inosine.

116. The composition of claim 98, wherein N1 and N2 comprise guanosine and N3 and N4 comprise cytidine, and wherein X1 and X2 each comprise inosine and no guanosine nucleosides.

117. The composition of claim 98, wherein N1 and N2 comprise cytidine and N3 and N4 comprise guanosine, and wherein X1 and X2 each comprise inosine and no guanosine nucleosides.

118. The composition of claim 98, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and wherein X1 and/or X2 each comprise at least one inosine.

119. The composition of claim 98, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and wherein X1 and X2 each comprise at least one inosine.

120. The composition of claim 98, wherein N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and wherein X1 and/or X2 each comprise at least one inosine.

121. The composition of claim 98, wherein N1 and N2 comprise inosine and N3 and N4 comprise cytidine, and wherein X1 and X2 comprise inosine and no guanosine nucleosides.

122. The composition of claim 98, wherein N1 and N2 comprise cytidine and N3 and N4 comprise inosine, and wherein X1 and X2 comprise inosine and no guanosine nucleosides.

123. The composition of claim 98, wherein X1 and X2 are each 12 nucleotides and comprise 1, 2, 3 or 4 inosine nucleosides

124. The composition of claim 98, wherein X1 and X2 are each 13 nucleotides and comprise 1, 2, 3, 4 or 5 inosine nucleosides.

125. The composition of claim 98, wherein X1 and X2 are each 14 nucleotides and comprise 1, 2, 3, 4, 5 or 6 inosine nucleosides.

126. The composition of claim 98, wherein X1 and X2 are each 15 nucleotides and comprise 1, 2, 3, 4, 5, 6, or 7 inosine nucleosides

127. The composition of claim 98, wherein X1 and X2 are each 16 nucleotides and each comprise 1, 2, 3, 4, 5, 6, 7, or 8 inosine nucleosides.

128. The composition of claim 98, wherein X1 and X2 are each 12 nucleotides and comprise at least 10%, 20%, 30% or 40% inosine nucleosides.

129. The composition of any one of claims 9-97, wherein the linker is a nucleotide linker or a non-nucleotide linker.

130. The composition of claim 129, wherein the linker is a non-nucleotide linker.

131. The composition of claim 129, wherein the linker is a nucleotide linker.

132. The composition of claim 131, wherein the nucleotide linker comprises a tetraloop, wherein the nucleotide sequence of the tetraloop is selected from the group consisting of:

(a) UNCG, wherein N = A, C, G, or U;

(b) GNRA, wherein N = A, C, G, or U, and wherein R = A or G;

(c) ANYA, wherein N = A, C, G, or U, and wherein Y = C or T;

(d) CUYG, wherein Y = C or T;

(e) UMAC, wherein M = A or C; and

(f) CUUG.

133. The composition of claim 131, wherein the nucleotide linker comprises the nucleotide sequence UUUGAU or UGUUU.

134. The composition of claim 132, wherein the sequence of the tetraloop is UUCG.

135. The composition of claim 132, wherein the sequence of the tetraloop is GAUC.

136. The composition of claim 133, wherein the nucleotide linker comprises the nucleotide sequence UUUGAU.

137. The composition of claim 133, wherein the nucleotide linker comprises the nucleotide sequence UGUUU.

138. The composition of any one of claims 98-128 and 130, wherein the non-nucleotide linker is selected from the group consisting of:

(a) an ethylene glycol linker; and

(b) an alkyl linker.

139. The composition of claim 138, wherein the non-nucleotide linker is a hexaethylene glycol linker.

140. The composition of claim 138, wherein the non-nucleotide linker is a C9 alkyl linker.

141. The composition of any one of claims 1-140, wherein the agonist comprises a 5' diphosphate moiety, or a derivative or analog thereof.

142. The composition of any one of claims 1-140, wherein the agonist comprises a 5' triphosphate moiety, or a derivative or analog thereof.

143. The composition of claim 141 or 142, wherein the derivative or analog thereof comprises a phosphate bioisostere is selected from: a phosphonate, a thiophosphonate, a phosphorothioate, a sulfate, a sulfonate, a sulfamate, a thiazolidinone, a carboxylate, a malonate, a boronic acid, a benzoxaborole, a boranophosphate, a squaramide.

144. The composition of any one of claims 1-143, wherein the agonist comprises a modified nucleotide, a modified nucleoside, or a modified nucleobase, or a combination thereof.

145. The composition of any one of claims 1-144, wherein the agonist comprises a modification to the internucleotide linkages or to the polynucleotide backbone.

146. The composition of any one of claims 1-145, wherein the agonist exhibits at least one or more of the following properties:

(a) specifically binds to one or more RLRs (e.g. RIG-1, MDA5 and/or LGP2);

(b) increases RLR-mediated cytokine production;

(c) increases RLR-mediated expression of interferon-stimulated genes (ISGs);

(d) increases RLR-dependent intracellular signaling;

(e) increases stability of the duplex;

(f) increases binding affinity to RLRs;

(g) decreases off-target binding;

(h) increases biological half-life;

(i) increases biodistribution and bioavailability;

(j) increases and/or enhances uptake into cells and/or tissues;

(k) decreases immunogenicity; and

(l) a combination of any of (a)-(k).

147. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the 5' most nucleotide of the agonist comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 1-36, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

148. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or

triphosphate moiety, or derivative or analog thereof, wherein the agonist comprises a sequence motif that provides at least one improved biological activity mediated by the RLR relative to an agonist that does not comprise the sequence motif, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

149. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, and wherein the agonist comprises the nucleotide sequence selected from the group consisting of SEQ ID NOs: 22, 23 and 25, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

150. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising at least one or more nucleotides comprising inosine which base pairs with cytidine, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 58 and 89, respectively;

(ii) SEQ ID NO: 59 and 89, respectively; and

(iii) SEQ ID NO: 61 and 91, respectively, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

151. A composition comprising:

(a) a virus-like particle; and

(b) at least one synthetic RLR agonist that specifically binds to RLRs, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a non-nucleotide linker, wherein the agonist comprises the formula 5'-($N_1$-$N_2$-$X_1$)-L-($X_2$-$N_3$-$N_4$)-3', wherein ($N_1$-$N_2$-$X_1$) comprises a first polynucleotide and ($X_2$-$N_3$-$N_4$) comprises a second polynucleotide, and wherein the first polynucleotide and the second polynucleotide comprise the nucleotide sequences selected from the group consisting of:

(i) SEQ ID NO: 37 and 68, respectively;

(ii) SEQ ID NO: 38 and 69, respectively;

(iii) SEQ ID NO: 39 and 70, respectively;

(iv) SEQ ID NO: 40 and 71, respectively;

(v) SEQ ID NO: 41 and 72, respectively;

(vi) SEQ ID NO: 42 and 73, respectively;

(vii) SEQ ID NO: 43 and 74, respectively;

(viii) SEQ ID NO: 44 and 75, respectively;

(ix) SEQ ID NO: 45 and 76, respectively;

(x) SEQ ID NO: 46 and 77, respectively;

(xi) SEQ ID NO: 47 and 78, respectively;

(xii) SEQ ID NO: 48 and 79, respectively;

(xiii) SEQ ID NO: 49 and 80, respectively;

(xiv) SEQ ID NO: 50 and 81, respectively;

(xv) SEQ ID NO: 51 and 82, respectively;

(xvi) SEQ ID NO: 52 and 83, respectively;

(xvii) SEQ ID NO: 53 and 84, respectively;

(xviii) SEQ ID NO: 54 and 85, respectively;

(xix) SEQ ID NO: 55 and 86, respectively;

(xx) SEQ ID NO: 56 and 87, respectively;

(xxi) SEQ ID NO: 57 and 88, respectively;

(xxii) SEQ ID NO: 58 and 89, respectively;

(xxiii) SEQ ID NO: 59 and 89, respectively;

(xxiv) SEQ ID NO: 60 and 90, respectively;

(xxv) SEQ ID NO: 61 and 91, respectively;

(xxvi) SEQ ID NO: 62 and 92, respectively;

(xxvii) SEQ ID NO: 63 and 91, respectively;

(xxviii) SEQ ID NO: 64 and 93, respectively;

(xxix) SEQ ID NO: 65 and 94, respectively;

(xxx) SEQ ID NO: 66 and 95, respectively;

(xxxi) SEQ ID NO: 67 and 96, respectively; and

(xxxii) SEQ ID NO: 63 and 97, respectively, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

152. The composition of any one of the preceding claims, wherein the nucleotide sequence comprising the agonist is not complementary to a genomic DNA sequence or mRNA sequence, wherein the RLR agonist does not participate in RNA interference, and wherein the RLR agonist does not silence gene expression.

153. The composition of any one of the preceding claims, wherein the virus-like particle lacks a lipoprotein-containing envelope.

154. The composition of any one of claims 1-152, wherein the virus-like particle is a recombinant virus-like particle.

155. The composition of claim 154, wherein the recombinant virus-like particle is selected from the group consisting of:

(a) recombinant proteins of Hepatitis B virus;

(b) recombinant proteins of measles virus;

(c) recombinant proteins of Sinbis virus;

(d) recombinant proteins of Rotavirus;

(e) recombinant proteins of Foot-and-Mouth-Disease virus;

(f) recombinant proteins of Retrovirus;

(g) recombinant proteins of Norwalk virus;

(h) recombinant proteins of human Papilloma virus;

(i) recombinant proteins of BK virus;

(j) recombinant proteins of bacteriophages;

(k) recombinant proteins of RNA-phages;

(1) recombinant proteins of Qβ-phage;

(m) recombinant proteins of GA-phage

(n) recombinant proteins of fr-phage;

(o) recombinant proteins of AP 205-phage;

(p) recombinant proteins of Ty; and

(q) fragments of any of the recombinant proteins from (a) to (p).

156. The composition of any one of claims 1-152, wherein the virus-like particle comprises recombinant proteins of an RNA-phage, wherein said RNA-phage is selected from the group consisting of: (a) bacteriophage Qβ; (b) bacteriophage R17; (c) bacteriophage fr; (d) bacteriophage GA; (e) bacteriophage SP; (f) bacteriophage MS2; (g) bacteriophage M11; (h) bacteriophage MX1; (i) bacteriophage NL95; (j) bacteriophage f2; (k) bacteriophage PP7; and (l) bacteriophage AP205.

157. The composition of claim 156, wherein the virus-like particle comprises recombinant proteins of bacteriophage Qβ.

158. The composition of any one of claims 1-152, wherein the virus-like particle comprises recombinant proteins, or fragments thereof, of RNA-phage Qβ.

159. A composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the RLR agonist comprises a ribonucleic acid (RNA) of 10-100 nucleotides in length, wherein the 5' most nucleotide of the RNA comprises a 5'diphosphate or triphosphate moiety, or derivative or analog thereof,

wherein the at least one RLR agonist is packaged in the virus-like particle.

160. A composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising a first polynucleotide connected to a second polynucleotide by a linker, wherein the first polynucleotide is sufficiently complementary to the second polynucleotide to form a duplex, wherein the duplex comprises less than 19 base pairs, wherein the 5' most nucleotide of the first oligonucleotide comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof, and wherein the agonist comprises a sequence motif selected from:

(i) a GT-repeat motif;

(ii) a GA-repeat motif;

(iii) a AUCG-repeat motif;

(iv) an AU-repeat motif;

(v) a dipyrimidine motif;

(vi) a dipurine motif;

(vii) a pyrimidine triplet motif;

(viii) a purine triplet motif;

(ix) a palindromic sequence motif; and

(x) a combination of any of (i)-(ix).,

wherein the at least one RLR agonist is packaged in the virus-like particle

161. A composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist comprises a blunt-ended, hairpin RNA comprising the formula:

5'-(N1-N2-X1)-L-(X2-N3-N4)-3', wherein

(i) (N1-N2-X1) comprises a first polynucleotide comprising linked nucleotides N1, N2 and X1;

(ii) (X2-N3-N4) comprises a second polynucleotide comprising linked nucleotides X2, N3 and N4;

(iii) N1, N2, N3 and N4 each comprise a single nucleotide comprising a nucleoside selected from the group consisting of: adenosine, guanosine, cytidine, 5-methyluridine, uridine and inosine;

(iv) N1 base pairs with N4;

(v) N2 base pairs with N3;

(vi) N1 comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof;

(vii) X1 comprises a sequence motif [AUCN5]x , wherein N5 is comprises guanosine or inosine, wherein x is an integer whose value indicates the number of sequence motifs, and wherein x= 2-4;

(viii) X2 comprises a sequence motif [CN6AU]y, wherein N6 comprises guanosine or inosine, wherein y is an integer whose value indicates the number of sequence motifs, and wherein y = 2-4;

(ix) L is a linker that operably links the first polynucleotide and the second polynucleotide,

optionally, wherein at least one of N1, N2, N3, and N4 is inosine, wherein the inosine nucleoside base pairs with cytidine in the hairpin RNA, and

wherein the at least one RLR agonist is packaged in the virus-like particle.

162. A composition comprising:

(a) a virus-like particle of an RNA-phage Qβ; and

(b) at least one synthetic RLR agonist that specifically binds to a RLR, wherein the agonist the nucleotide sequence of SEQ ID NO: 23, and wherein the 5' most nucleotide of the agonist comprises a 5' diphosphate or triphosphate moiety, or derivative or analog thereof,

wherein the at least one RLR agonist is packaged in the virus-like particle.

163. The composition of any one of claims 159-162, wherein the virus-like particle comprises RNA-phage Qβ coat proteins each having the amino acid sequence of SEQ ID NO: 112.

164 The composition of any one of claims 159-162, wherein the virus-like particle comprises RNA-phage Qβ coat proteins each having an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%,or 99% identical to SEQ ID NO: 112.

165. The composition of any one of the preceding claims, wherein the RLR agonist is non-covalently bound to the virus-like particle.

166. The composition of any one of claims 1-164, wherein the RLR agonist is bound to a virus-like particle site selected from the group consisting of an oligonucleotide binding site, a DNA binding site and an RNA binding site.

167. The composition of claim 166, wherein the virus-like particle site comprises an arginine-rich repeat.

168. The composition of any one of the preceding claims, further comprising at least one antigen or antigenic determinant bound to the virus-like particle.

169. The composition of claim 168, wherein the at least one antigen or antigenic determinant is bound to the virus-like particle by at least one covalent bond.

170. The composition of claim 168, wherein the at least one antigen or antigenic determinant is bound to the virus-like particle by a non-peptide bond.

171. The composition of claim 168, wherein the antigen or antigenic determinant is fused to the virus-like particle.

172. The composition of claim 168, wherein the virus-like particle comprises at least one first attachment site and wherein the antigen or antigenic determinant comprises at least one second attachment site selected from the group consisting of (a) an attachment site not naturally occurring within the antigen or antigenic determinant; and (b) an attachment site naturally occurring within the antigen or antigenic determinant, and

wherein the binding of the antigen or antigenic determinant to the virus-like particle is effected through association between the first attachment site and the second attachment site, optionally wherein the association is through at least one non-peptide bond.

173. The composition of claim 172, wherein the first attachment site comprises an amino group or a lysine residue, and wherein the second attachment site comprises a sulfhydryl group or a cysteine residue.

174. A pharmaceutical composition for stimulating an immune response, treating or delaying progression of a cancer, or reducing or inhibiting tumor growth in a subject in need thereof, comprising the composition of any one of claim 1-173, and a pharmaceutically acceptable carrier.

175. The pharmaceutical composition of claim 174, wherein the composition is formulated in a polyethylenimine (PEI) carrier.

176. The pharmaceutical composition of claim 175, wherein the PEI carrier is JetPEI®.

177. A method to increase RLR-mediated production of one or more cytokines in a cell, the method comprising contacting the cell with the composition of any one of claims 1-173, wherein the agonist increases RLR-mediated cytokine production in a cell.

178. A method to increase RLR-mediated expression of one or more interferon-stimulated genes in a cell, the method comprising contacting the cell with the composition of any one of claims 1-173, wherein the agonist increases RLR-mediated expression of one or more interferon-stimulated genes in a cell.

179. A method to increase RLR-dependent intracellular signaling in a cell, the method comprising contacting the cell with the composition of any one of claims 1-173, wherein the agonist increases RLR-dependent intracellular signaling.

180. A method of stimulating an immune response in a subject, the method comprising administering to the subject an effective amount of the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176.

181. A method of treating or delaying progression of a cancer in a subject, the method comprising administering to the subject an effective amount of the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176.

182. A method of reducing or inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject an effective amount of the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176.

183. A method for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, the method comprising administering to the subject an effective amount of the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176, wherein the agonist, increases RLR-mediated production of one or more cytokines in a cell, increases RLR-mediated expression of one or more interferon-stimulated genes in a cell, and or increases RLR-dependent intracellular signaling in a cell, thereby stimulating the immune response, treating or delaying progression of the cancer, or inhibiting growth of the tumor.

184. The method of any one of claims 180-183, wherein the composition is administered in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a cytokine, an activator or agonist of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof.

185. The method of claim 184, wherein the composition is administered preceding or subsequent to administration of the one or more additional therapeutic agents or wherein the one

or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the agonist or pharmaceutical composition.

186. The method of claim 184 or 185, wherein the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist, a TIM-3 antagonist, a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, a KIR antagonist, a LAG-3 antagonist, a Toll-like receptor 3 (TLR3) agonist, a Toll-like receptor 7 (TLR7) agonist, a Toll-like receptor 9 (TLR9) agonist.

187. The method of claim 184 or 185, wherein the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g, antibody, or antigen binding portion thereof) that specifically binds to CD137 (4-1BB).

188. The method of claim 184 or 185, wherein the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD134 (OX40).

189. The method of claim 186, wherein the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist.

190. The method of claim 189, wherein the PD-1/PD-L1 antagonist is selected from the group consisting of: PDR001, KEYTRUDA® (pembrolizumab), OPDIVO® (nivolumab), pidilizumab, MEDI0680, REGN2810, TSR-042, PF-06801591, and AMP-224.

191. The method of claim 189, wherein the PD-1/PD-L1 antagonist is selected from the group consisting of: FAZ053, TENCENTRIQ® (atezolizumab), BAVENCIO® (avelumab), IMFINZI® (durvalumab), and BMS-936559.

192. The method of claim 186, wherein the one or more additional therapeutic agents is a TIM-3 antagonist.

193. The method of claim 186, wherein the one or more additional therapeutic agents is a VISTA antagonist.

194. The method of claim 186, wherein the one or more additional therapeutic agents is an adenosine A2AR antagonist.

195. The method of claim 186, wherein the one or more additional therapeutic agents is a B7-H3 antagonist.

196. The method of claim 186, wherein the one or more additional therapeutic agents is a B7-H4 antagonist.

197. The method of claim 186, wherein the one or more additional therapeutic agents is a BTLA antagonist.

198. The method of claim 186, wherein the one or more additional therapeutic agents is a CTLA-4 antagonist.

199. The method of claim 186, wherein the one or more additional therapeutic agents is a IDO antagonist.

200. The method of claim 186, wherein the one or more additional therapeutic agents is a KIR antagonist.

201. The method of claim 186, wherein the one or more additional therapeutic agents is a LAG-3 antagonist.

202. The method of claim 186, wherein the one or more additional therapeutic agents is a Toll-like receptor 3 (TLR3) agonist.

203. The method of claim 202, wherein the TLR3 agonist is polyinosinic:polycytidylic acid (poly I:C).

204. The method of claim 202, wherein the TLR3 agonist is HILTONOL® (poly ICLC).

205. The method of claim 202, wherein the TLR3 agonist is polyadenylic-polyuridylic acid (poly A:U).

206. The method of claim 202, wherein the TLR3 agonist is RIBOXXIM® (RGIC®100).

207. The method of claim 202, wherein the TLR3 agonist is RIBOXXON® (RGIC®50 bioconjugate).

208. The method of claim 202, wherein the TLR3 agonist is RIBOXXOL® (RGIC®50).

209. The method of claim 186, wherein the one or more additional therapeutic agents is a Toll-like receptor 7 (TLR7) agonist.

210. The method of claim 209, wherein the TLR7 agonist is GS-9620 (Vesatolimod).

211. The method of claim 209, wherein the TLR7 agonist is imiquimod (ALDARA$^{TM}$).

212. The method of claim 209, wherein the TLR7 agonist is resiquimod (R-848).

213. The method of claim 186, wherein the one or more additional therapeutic agents is a Toll-like receptor 9 (TLR9) agonist.

214. The method of claim 213, wherein the TLR9 agonist is a CpG oligodeoxynucleotide (CpG ODN).

215. The method of claim 214, wherein the CpG ODN is a Class A CpG ODN (CpG-A ODN).

216. The method of claim 214, wherein the CpG ODN is a Class B CpG ODN (CpG-B ODN).

217. The method of claim 214, wherein the CpG ODN is a Class C CpG ODN (CpG-C ODN).

218. Use of the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176, for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, optionally for use in combination with one or more additional therapeutic agents.

219. Use of the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176, in the manufacture of a medicament for stimulating an immune response, treating or delaying progression of a cancer, or inhibiting tumor growth in a subject in need thereof, optionally for use in combination with one or more additional therapeutic agents.

220. A kit comprising the composition of any one of claims 1-173, or the pharmaceutical composition of any one of claims 174-176 and instructions for use in stimulating an immune response in a subject, or treating or delaying progression of a cancer, or inhibiting tumor growth in a subject, optionally with instructions for use in combination with one or more additional therapeutic agents.

221. The use of claim 218 or kit of claim 220, wherein the agonist or pharmaceutical composition is administered in combination with one or more additional therapeutic agents, wherein the one or more additional therapeutic agents is selected from the group consisting of: a chemotherapy, a targeted anti-cancer therapy, an oncolytic drug, a cell death-inducing agent, an opsonizing agent (e.g., an opsonizing antibody) a cytotoxic agent, an immune-based therapy, a

cytokine, an activator of a costimulatory molecule, an inhibitor of an inhibitory molecule, a vaccine, a cellular immunotherapy, or a combination thereof.

222. The use or kit of claim 221, wherein the agonist or pharmaceutical composition is administered preceding or subsequent to administration of the one or more additional therapeutic agents or wherein the one or more additional therapeutic agents is administered concurrently with, preceding or subsequent to the administration of the agonist or pharmaceutical composition.

223. The use of any one of claims 218, 219, 221 or 222 or the kit of claims 220-222, wherein the one or more additional therapeutic agents is a PD-1/PD-L1 antagonist, a TIM-3 antagonist, a VISTA antagonist, an adenosine A2AR antagonist, a B7-H3 antagonist, a B7-H4 antagonist, a BTLA antagonist, a CTLA-4 antagonist, an IDO antagonist, a KIR antagonist, a LAG-3 antagonist, a Toll-like receptor 3 (TLR3) agonist, a Toll-like receptor 7 (TLR7) agonist, a Toll-like receptor 9 (TLR9) agonist.

224. The use of any one of claims 218, 219, 221 or 222 or the kit of claims 220-222, wherein the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g, antibody, or antigen binding portion thereof) that specifically binds to CD137 (4-1BB).

225. The use of any one of claims 218, 219, 221 or 222 or the kit of claims 220-222, wherein the one or more additional therapeutic agents is an agonist comprising an polypeptide (e.g., antibody, or antigen binding portion thereof) that specifically binds to CD134 (OX40).

226. A method of producing the composition of any one of claims 1-173, comprising:

(a) disassembling the virus-like particle;

(b) adding the RLR agonist; and

(c) reassembling the virus-like particle.

227. The method of claim 226, further comprising removing nucleic acids of the disassembled virus-like particle.

228. The method of claim 226 or 227, further comprising purifying the composition after reassembly.

229. The method of any one of claims 226-228, comprising (d) binding an antigen or antigenic determinant to the virus-like particle.

230. The method of claim 229, wherein the antigen or antigenic determinant is bound to the virus-like particle before disassembling the virus-like particle.

231. The method of claim 229, wherein the antigen or antigenic determinant is bound to the virus-like particle after reassembling the virus-like particle.

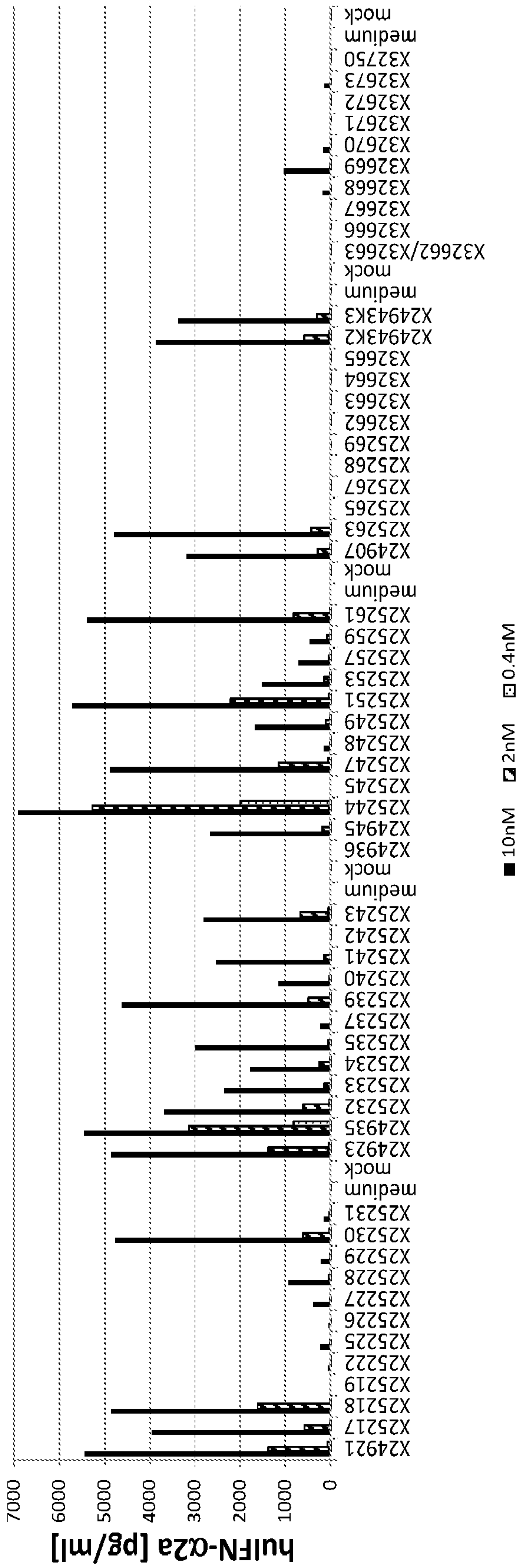

huIFN-α2a [pg/ml]
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5000
4000
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X25225
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X32666
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FIG. 1

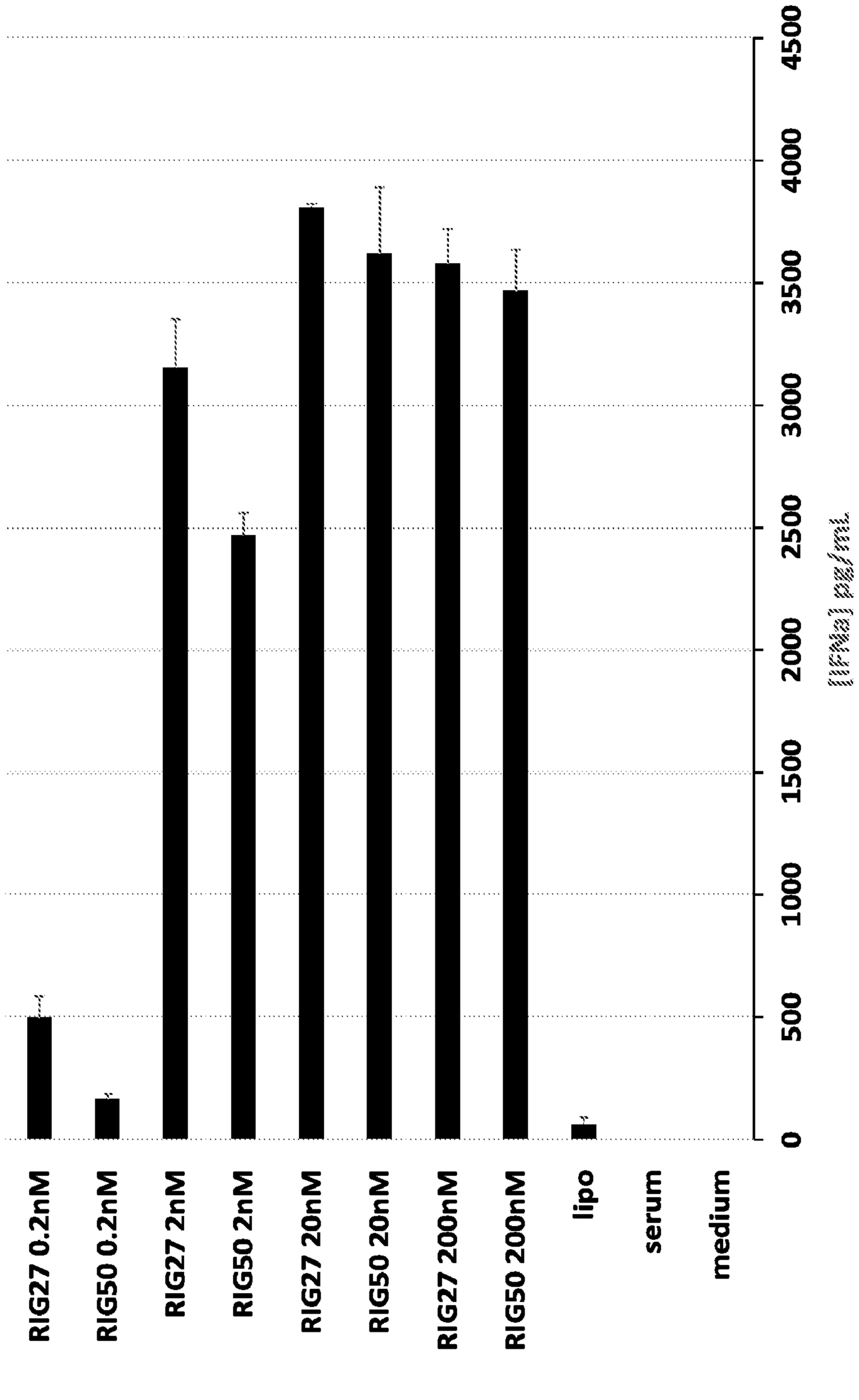

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RIG50 0.2nM
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RIG50 20nM
RIG27 200nM
RIG50 200nM
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serum
medium

FIG. 2

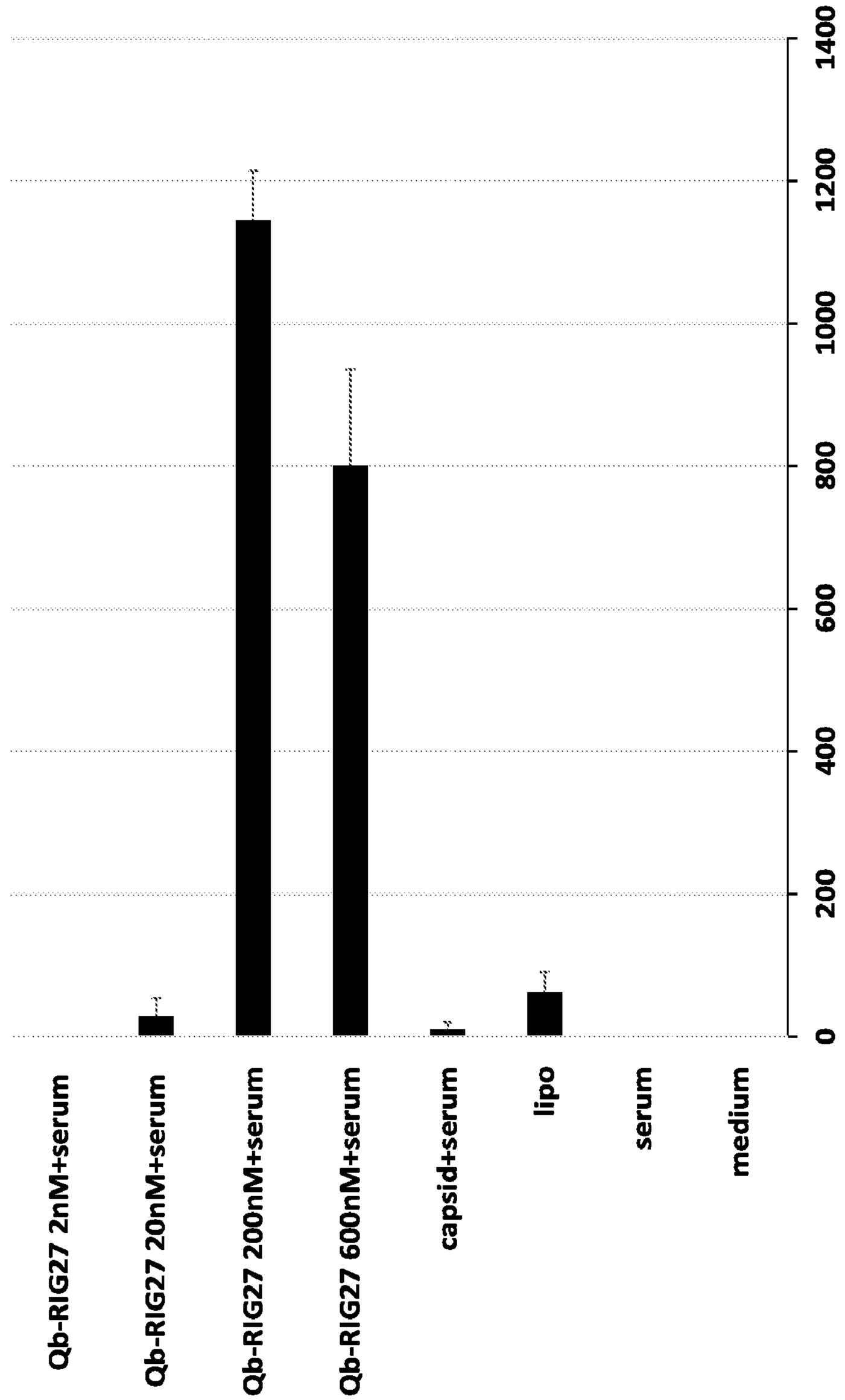

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1000
800
600
400
200
0
Urinal pg/mL
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Qb-RIG27 20nM+serum
Qb-RIG27 200nM+serum
Qb-RIG27 600nM+serum
capsid+serum
lipo
serum
medium

FIG. 3

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/057099

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/117 C12N7/00 A61K31/713 A61P37/04
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K A61P C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search, EMBASE, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2014/169049 A1 (UNIV DUKE [US])<br>16 October 2014 (2014-10-16)<br>page 7 - page 9; claims 1,12,13,21; figure 1<br>page 23 - page 24<br>----- | 1-12 |
| X<br><br><br><br>Y | WO 2018/172546 A1 (RIGONTEC GMBH [DE])<br>27 September 2018 (2018-09-27)<br><br><br>page 22 - page 23; claims 10-24<br><br>-----<br>-/-- | 1-8,<br>10-12,<br>146,174,<br>177-183,<br>218-220<br>9,<br>13-145,<br>147-173,<br>175,176,<br>184-217,<br>221-231 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 February 2021 | 23/02/2021 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Bucka, Alexander |

1

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/057099

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 03/024481 A2 (CYTOS BIOTECHNOLOGY AG [CH]; BACHMANN MARTIN F [CH] ET AL.)<br>27 March 2003 (2003-03-27)<br>claims 1-6,12; examples 2-4, 7, 9, 12-16<br>----- | 1-231 |
| Y | WO 2009/141146 A1 (HARTMANN GUNTHER [DE])<br>26 November 2009 (2009-11-26)<br>claims 1-6,13-19<br>----- | 1-231 |
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| Y | WO 2019/197965 A1 (CHECKMATE PHARMACEUTICALS [US])<br>17 October 2019 (2019-10-17)<br>claims 1,5,17,18,22-26<br>----- | 1-231 |
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**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

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2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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