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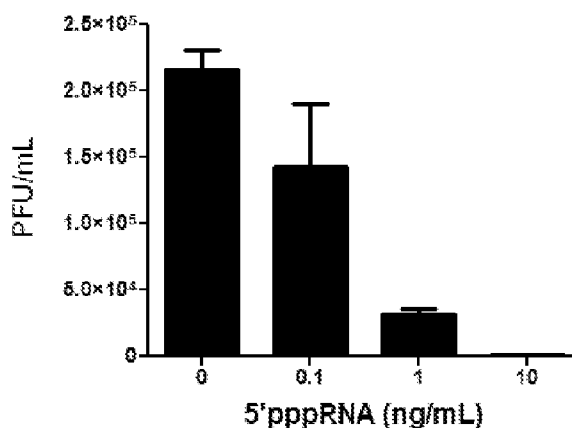
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(54) Title: 5'-TRIPHOSPHATE OLIGORIBONUCLEOTIDES

Figure 4D



(57) Abstract: Disclosed are 5'-triphosphate oligoribonucleotides, pharmaceutical compositions comprising said 5'-triphosphate oligoribonucleotides, and methods of using said 5'-triphosphate oligoribonucleotides to treat viral infections.

TITLE

5'-TRIPHOSPHATE OLIGORIBONUCLEOTIDES

FIELD

Generally, the field is RNA-based therapeutic molecules. More specifically, the field is 5'-triphosphate oligoribonucleotide immune system agonists and pharmaceutical compositions comprising the same.

PRIORITY CLAIM

This application claims the benefit of US Provisional Application 61/763,367, filed 11 February 2013 and is hereby incorporated by reference in its entirety.

BACKGROUND

The innate immune system has evolved numerous molecular sensors and signaling pathways to detect, contain and clear viral infections (Takeuchi O and Akira S *Immunol Rev* **227**, 75-86 (2009); Yoneyama M and Fujita T, *Rev Med Virol* **20**, 4-22 (2010); Wilkins C and Gale M *Curr Opin Immunol* **22**, 41-47 (2010); and Brennan K and Bowie AG *Curr Opin Microbiol* **13**, 503-507 (2010); all of which are incorporated by reference herein.) Viruses are sensed by a subset of pattern recognition receptors (PRRs) that recognize evolutionarily conserved structures known as pathogen-associated molecular patterns (PAMPs). Classically, viral nucleic acids are the predominant PAMPs detected by these receptors during infection. These sensing steps contribute to the activation of signaling cascades that culminate in the early production of antiviral effector molecules, cytokines and chemokines responsible for the inhibition of viral replication and the induction of adaptive immune responses (Takeuchi O and Akira S *Cell* **140**, 805-820 (2010), Liu SY *et al*, *Curr Opin Immunol* **23**, 57-64 (2011); and Akira S *et al*, *Cell* **124**, 783-801 (2006); all of which are incorporated by reference herein). In addition to the nucleic acid sensing by a subset of endosome-associated Toll-like receptors (TLR),

viral RNA structures within the cytoplasm are recognized by members of the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) family, including the three DExD/H box RNA helicases RIG-I, Mda5 and LGP-2 (Kumar H *et al*, *Int Rev Immunol* **30**, 16-34 (2011); Loo YM and Gale M, *Immunity* **34**, 680-692 (2011); Belgnaoui SM *et al*, *Curr Opin Immunol* **23**, 564-572 (2011); Beutler BE, *Blood* **113**, 1399-1407 (2009); Kawai T and Akira S, *Immunity* **34**, 637-650 (2011); all of which are incorporated by reference herein.)

RIG-I is a cytosolic multidomain protein that detects viral RNA through its helicase domain (Jiang F *et al*, *Nature* **479**, 423-427 (2011) and Yoneyama M and Fujita T, *J Biol Chem* **282**, 15315-15318 (2007); both of which are incorporated by reference herein). In addition to its RNA sensing domain, RIG-I also possesses an effector caspase activation and recruitment domain (CARD) that interacts with the mitochondrial adaptor MAVS, also known as VISA, IPS-1, and Cardif (Kawai T *et al*, *Nat Immunol* **6**, 981-988 (2005) and Meylan E *et al*, *Nature* **437**, 1167-1172 (2005), both of which are incorporated by reference herein.) Viral RNA binding alters RIG-I conformation from an auto-inhibitory state to an open conformation exposing the CARD domain, resulting in RIG-I activation which is characterized by ATP hydrolysis and ATP-driven translocation of RNA (Schlee M *et al*, *Immunity* **31**, 25-34 (2009); Kowlinski E *et al*, *Cell* **147**, 423-435 (2011); and Myong S *et al*, *Science* **323**, 1070-1074 (2011); all of which are incorporated by reference herein). Activation of RIG-I also allows ubiquitination and/or binding to polyubiquitin. In recent studies, polyubiquitin binding has been shown to induce the formation of RIG-I tetramers that activate downstream signaling by inducing the formation of prion-like fibrils comprising the MAVS adaptor (Jiang X *et al*, *Immunity* **36**, 959-973 (2012); incorporated by reference herein). MAVS then triggers the activation of IRF3, IRF7 and NF- κ B through the IKK-related serine kinases TBK1 and IKK ϵ (Sharma S *et al*, *Science* **300**, 1148-1151 (2003); Xu LG *et al*, *Molecular Cell* **19**, 727-740 (2005); and Seth RB *et al*, *Cell* **122**, 669-682 (2005); all of which are incorporated by reference herein). This in turn leads to the expression of type I interferons (IFN β and IFN α), as well

as pro-inflammatory cytokines and anti-viral factors (Tamassia N *et al*, *J Immunol* **181**, 6563-6573 (2008) and Kawai T and Akira S, *Ann NY Acad Sci* **1143**, 1-20 (2008); both of which are incorporated by reference herein.) A secondary response involving the induction of IFN stimulated genes (ISGs) is induced by the binding of IFN to its cognate receptor (IFN α / β R). This triggers the JAK-STAT pathway to amplify the antiviral immune response (Wang BX and Fish EN *Trends Immunol* **33**, 190-197 (2012); Nakhaei P *et al*, Activation of Interferon Gene Expression Through Toll-like Receptor-dependent and – independent Pathways, in *The Interferons*, Wiley-VCH Verlag GmbH and Co KGaA, Weinheim FRG (2006); Sadler AJ and Williams BR, *Nat Rev Immunol* **8**, 559-568 (2008); and Schoggins JW *et al*, *Nature* **472**, 481-485 (2011); all of which are incorporated by reference herein.)

The nature of the ligand recognized by RIG-I has been the subject of intense study given that PAMPs are the initial triggers of the antiviral immune response. In vitro synthesized RNA carrying an exposed 5' terminal triphosphate (5'ppp) moiety was identified as a RIG-I agonist (Hornung V *et al*, *Science* **314**, 994-997 (2006); Pichlmair A *et al*, *Science* **314**, 997-1001 (2006); and Kim DH *et al*, *Nat Biotechnol* **22**, 321-325 (2004); all of which are incorporated by reference herein). The 5'ppp moiety is added to the end of all viral and eukaryotic RNA molecules generated by RNA polymerization. However, in eukaryotic cells, RNA processing in the nucleus cleaves the 5'ppp end and the RNA is capped prior to release into the cytoplasm. The eukaryotic immune system evolved the ability to distinguish viral 'non-self' 5'ppp RNA from cellular 'self' RNA through RIG-I (Fujita T, *Immunity* **31**, 4-5 (2009); incorporated by reference herein). Further characterization of RIG-I ligand structure indicated that blunt base pairing at the 5' end of the RNA and a minimum double strand (ds) length of 20 nucleotides were also important for RIG-I signaling (Schlee M and G Hartmann, *Molecular Therapy* **18**, 1254-1262 (2010); incorporated by reference herein). Further studies indicated that a dsRNA length of less than 300 base pairs led to RIG-I activation but a dsRNA length of more

than 2000bp lacking a 5'ppp (as is the case with poly I:C) failed to activate RIG-I. (Kato H *et al*, *J Exp Med* **205**, 1601-1610 (2008); incorporated by reference herein).

RNA extracted from virally infected cells, specifically viral RNA genomes or viral replicative intermediates, was also shown to activate RIG-I (Baum A *et al*, *Proc Natl Acad Sci USA* **107**, 16303-16308 (2010); Rehwinkel J and Sousa CRE, *Science* **327**, 284-286 (2010); and Rehwinkel J *et al*, *Cell* **140**, 397-408 (2010); all of which are incorporated by reference herein). Interestingly, the highly conserved 5' and 3' untranslated regions (UTRs) of negative single strand RNA virus genomes display high base pair complementarity and the panhandle structure theoretically formed by the viral genome meets the requirements for RIG-I recognition. The elucidation of the crystal structure of RIG-I highlighted the molecular interactions between RIG-I and 5'ppp-dsRNA (Cui S *et al*, *Molecular Cell* **29**, 169-179 (2008); incorporated by reference herein), providing a structural basis for the conformational changes involved in exposing the CARD domain for effective downstream signaling.

SUMMARY

Disclosed herein is a oligoribonucleotide derived from the 5' and 3'UTRs of the VSV genome (SEQ ID NO: 1) synthesized with a triphosphate group at its 5' end (5'ppp-SEQ ID NO: 1). The 5'ppp-SEQ ID NO: 1 activates the RIG-I signaling pathway and triggers a robust antiviral response that interferes with infection by several pathogenic viruses, including Dengue, HCV, HIV-1 and H1N1 Influenza A/PR/8/34. Furthermore, intravenous delivery of 5'ppp-SEQ ID NO: 1 stimulates an antiviral state in vivo that protects mice from lethal influenza virus challenge.

Also disclosed are modified variants of 5'ppp-SEQ ID NO: 1 that include locked nucleic acids, G-clamp nucleotides, nucleotide base analogs, terminal cap moieties, phosphate backbone modifications, conjugates, and the like.

Also disclosed are pharmaceutical compositions comprising 5'ppp-SEQ ID NO: 1 and/or a modified variant thereof and a pharmaceutically acceptable carrier that acts as

a transfection reagent such as a lipid based carrier, a polymer based carrier, a cyclodextrin based carrier, a protein based carrier and the like.

Also disclosed are methods of treating a viral infection in a subject by administering one or more of the pharmaceutical compositions to a subject.

DESCRIPTION OF THE DRAWINGS

The term "5'pppRNA," used in the figures is equivalent to the term "5'ppp-SEQ ID NO: 1" used in the text and these terms may be used interchangeably.

Figure 1A through Figure 1D show that 5'ppp-SEQ ID NO: 1 stimulates an antiviral and inflammatory response in lung epithelial A549 cells.

Figure 1A is a 2-D representation of 5'ppp-SEQ ID NO: 1 (top panel) and an image of a gel showing that the in vitro transcription product of 5'-ppp-SEQ ID NO: 1 is a single product degraded by RNase I.

Figure 1B is an image of an immunoblot in which 5'ppp-SEQ ID NO: 1 or a homologous control of SEQ ID NO: 1 alone (lacking the 5'-triphosphate) was mixed with Lipofectamine RNAiMax® and transfected at the RNA concentrations indicated (0.1 - 500ng/ml) into A549 cells. At 8 hours post treatment, whole cell extracts were prepared, resolved by SDS-page and immunoblotted with antibodies specific for IRF3 pSer396, IRF3, ISG56, NOXA, cleaved caspase 3, PARP and β -actin as indicated. Results are from a representative experiment; all immunoblots are from the same samples.

Figure 1C is an image of immunoblots of whole cell extracts of A549 cells transfected with 10 ng/ml 5'ppp-SEQ ID NO: 1 and probed with antibodies specific to the indicated proteins. Whole cell extracts were prepared at different times after transfection (0-48 hours), electrophoresed by SDS-PAGE and probed with antibodies specific for IRF3 pSer-396, IRF3, IRF7, STAT1 pTyr-701, STAT1, ISG56, RIG-I, I κ B α pSer-32, I κ B α and β -actin. All immunoblots are from the same samples. To detect IRF3 dimerization (top panel,) whole cell extracts were resolved by native-PAGE and analyzed by immunoblotting for IRF3.

Figure 1D is a set of two bar graphs showing the results of ELISA assays to detect IFN β and IFN α in cell culture supernatants at the indicated times. Error bars represent SEM from two independent samples.

Figures 2A-2D demonstrate that the induction of the interferon response by 5'ppp-SEQ ID NO: 1 is dependent on functional RIG-I signaling

Figure 2A is a set of two bar graphs showing the fold induction of IFN β and IFN α 4 in wild type and RIG-I^{-/-} mouse endothelial fibroblasts (MEF's) by 5'ppp-SEQ ID NO: 1 and a constitutively active form of RIG-I (Δ RIG-I) (100ng). MEF's were co-transfected with an IFN α 4 or IFN β promoter reporter plasmid (200ng) along with 5'ppp-SEQ ID NO: 1 (500ng/ml) or an expression plasmids encoding Δ RIG-I. An IRF-7 expression plasmid (100ng) was added for transactivation of the IFN α 4 promoter. Luciferase activity was analyzed 24 hours post transfection by the Dual-Luciferase Reporter assay. Relative luciferase activity was measured as fold induction relative to the basal level of reporter gene. Error bars represent SEM from nine replicates performed in three independent experiments.

Figure 2B is a bar graph showing the induction of IFN β in MDA5^{-/-}, TLR3^{-/-}, TLR7^{-/-} and RIG-I^{-/-} MEFs by 5'ppp-SEQ ID NO: 1 and Δ RIG-I. MEFs were co-transfected with IFN β promoter reporter plasmid (200ng) along with 5'ppp-SEQ ID NO: 1 (500ng/ml). Luciferase activity was analyzed 24h post-transfection by the Dual-Luciferase Reporter assay. Relative luciferase activity was measured as fold induction relative to the basal level of reporter gene. Promoter activity in the knockout MEFs was then normalized against the activity in their respective wild type MEF's to obtain the percentage of activation. Error bars represent SEM from nine replicates performed in three independent experiments.

Figure 2C is an image of a set of immunoblots of whole cell extracts of A549 cells and A549 cells deficient in MAVS expression. 5'ppp-SEQ ID NO: 1 was transfected in control A549 and MAVS shRNA A549 cells at different concentrations (0, 0.1, 1, 10, 100ng/ml). At 8 hours after treatment, whole cell extracts were analyzed by SDS-PAGE,

blotted, and probed with antibodies specific for pIRF3 Ser-396, IRF3, pSTAT1 Tyr 701, STAT1, ISG56, MAVS (VISA), and β -Actin. Results are from a representative experiment; all immunoblots are from the same samples.

Figure 2D is an image of an immunoblot of whole cell extracts of A549 cells, A549 cells transfected with siRNA that silences RIG-I expression, and an irrelevant negative control siRNA. Cells were transfected with 5'-ppp-SEQ ID NO: 1 as indicated and whole cell extracts were analyzed by SDS-PAGE, blotted, and probed with antibodies specific for the indicated proteins.

Figures 3A-3E depict 5'-ppp-SEQ ID NO: 1 acting as a broad-spectrum antiviral agent.

Figure 3A is a set of three bar graphs showing the percent of cells infected with VSV, Dengue, and Vaccinia as indicated and treated with 5'-ppp-SEQ ID NO: 1 as indicated. A549 cells were transfected with 10ng/ml 5'-ppp-SEQ ID NO: 1 24 hours prior to infection and infected with VSV Δ 51-GFP (MOI = 0.1), Dengue virus (MOI = 0.1), and Vaccinia-GFP virus (MOI = 5), respectively. Percentage of infected cells was determined 24 hours post-infection by flow cytometry analysis of GFP expression (VSV-GFP and Vaccinia-GFP) or intracellular staining of DENV E protein expression (Dengue virus). Data are from a representative experiment performed in triplicate. Error bars represent the standard deviation.

Figure 3B is a set of six flow cytometry plots showing the results of CD14⁺ and CD14⁻ human PBMCs treated with 5'-ppp-SEQ ID NO: 1 as indicated and infected with Dengue virus as indicated. PBMCs were transfected with 100ng/ml 5'-ppp-SEQ ID NO: 1 24 hours prior to infection with dengue virus at an MOI of 5. At 24 hours post-infection, the percentage of Dengue infected CD14⁺ and CD14⁻ cells was evaluated by intracellular staining of DENV E protein expression by flow cytometry. Data are from a representative experiment performed in triplicate. Error bars represent the standard deviation.

Figure 3C is a bar graph showing the results of human PBMC's infected with DENV2 as indicated, treated with 5'-ppp-SEQ ID NO: 1 (called 5'-pppVSV in this figure),

and treated with the Lyovect[®] transfection agent as indicated. Human PBMCs from three different donors were transfected with 100 ng/ml 5'ppp-SEQ ID NO: 1 prior to infection with Dengue virus at an MOI of 5. The percentage of Dengue infected cells in the CD14⁺ population was evaluated by intracellular staining of DENV E protein expression using flow cytometry. Data are from an experiment performed in triplicate on three different patients. Error bars represent the standard deviation.

Figure 3D is a set of three flow cytometry histograms depicting the results of human CD4⁺ T cells infected with HIV-GMP as indicated and treated with 5'ppp-SEQ ID NO: 1 as indicated. CD4⁺ T cells were isolated from human PBMCs and activated with anti-CD3 and anti-CD28 antibodies. Cells were incubated in the presence or absence of supernatant from 5'ppp-SEQ ID NO: 1-treated monocytes for 4 hours and infected with HIV-GFP (MOI = 0.1) for 48 hours. The percentage of HIV infected, activated CD4⁺ T cells (GFP positive) was assessed by flow cytometry.

Figure 3E is an image of an immunoblot of whole cell extracts of Huh7 and Huh7.5 cells transfected with 5'ppp-SEQ ID NO: 1 (10 ng/ml) as indicated and infected with Hepatitis C Virus (HCV) 24 hours after treatment with 5'ppp-SEQ ID NO: 1 as indicated. At 48 hours post-infection, analyzed by SDS-PAGE, blotted, and probed with antibodies specific for the HCV viral protein NS3 and IFIT1 as well as β -actin.

Figures 4A-4F depict 5'ppp-SEQ ID NO: 1 as an inhibitor of H1N1 Influenza replication in vitro.

Figure 4A is an image of an immunoblot of whole cell extracts from A549 cells probed with antibodies to the indicated proteins. A549 cells were treated with 5'ppp-SEQ ID NO: 1 (10 ng/ml) as indicated. At 24 hours post-treatment, cells were infected with an increasing MOI of A/PR8/34 H1N1 Influenza virus (0.02 MOI, 0.2 MOI, or 2 MOI) for 24 hours. Whole cell extracts were run on an SDS-PAGE gel and immunoblotted to detect expression of the influenza viral proteins NS1, ISG56, and β -actin.

Figure 4B is a bar graph depicting viral titers in the cell culture supernatants from the samples shown in Figure 7A. Viral titer was determined by plaque assay. Error bars represent the standard error of the mean from two independent samples.

Figure 4C is an image of an immunoblot of whole cell extracts of A549 cells probed with antibodies to the indicated proteins. A549 cells were treated with increasing concentrations of 5'ppp-SEQ ID NO: 1 (0.1 ng/ml to 10 ng/ml) for 24 hours prior to infection with 0.2 MOI of influenza. Whole cell extracts were run on an SDS-PAGE gel and immunoblotted to detect expression of viral proteins NS1, ISG56, and β -Actin.

Figure 4D is a bar graph depicting the viral titers in cell culture supernatants from the samples shown in Figure 6C. Viral titer was determined by plaque assay. Error bars represent SEM from two independent samples.

Figure 4E is an image of an immunoblot of whole cell extracts of A549 cells probed with antibodies to the indicated proteins. A549 cells were treated with 5'ppp-SEQ ID NO: 1 (10 ng/ml) both before and after infection with 0.02 MOI of influenza as indicated on the legend above the gel (numbers are in days.) Whole cell extracts were run on an SDS-PAGE gel and immunoblotted to detect expression of the indicated proteins.

Figure 4F is an image of an immunoblot of whole cell extracts of A549 cells transfected with a control siRNA, RIG-I siRNA or IFN α / β receptor siRNA and then treated with 5'-ppp-SEQ ID NO: 1 at 10 ng/ml as indicated and infected with Influenza at 0.2 MOI as indicated. The whole cell extracts were prepared 24 hours after infection, run on an SDS-PAGE gel, and immunoblotted to detect expression the indicated proteins.

Figure 7G is an immunoblot of whole cell extracts of A549 cells transfected with a control siRNA or an IFN α / β R siRNA and then treated with 5'-ppp-SEQ ID NO: 1 at 10 ng/ml or IFN α -2b at 100 IU/ml) for 24 hours. The whole cell extracts were prepared 24 hours after infection, run on an SDS-PAGE gel, and immunoblotted to detect expression the indicated proteins.

Figures 5A-5I demonstrate that 5'ppp-SEQ ID NO: 1 activates innate immunity and protects mice from lethal influenza infection *in vivo*. All mice treated with 5'ppp-SEQ ID NO: 1 were injected intravenously with 25 µg of 5'ppp-SEQ ID NO: 1 in complex with In vivo Jet-PEI®. Statistical analysis was performed by Student's t test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, not statistically significant).

Figure 5A is a plot depicting the percent survival over time of mice treated with 5'ppp-SEQ ID NO: 1 one day prior to infection with 500 PFU of influenza relative to non-treated (NT) mice as indicated.

Figure 5B is a plot depicting the percent weight loss over time of mice treated with 5'ppp-SEQ ID NO: 1 one day prior to infection with 500 PFU of influenza relative to non-treated (NT) mice as indicated.

Figure 5C is a bar graph depicting the influenza viral titer over time in the lung of mice treated with 5'ppp-SEQ ID NO: 1 one day prior to infection with 500 PFU of influenza relative to non-treated (NT) mice as indicated. Viral titer was measured by plaque assay. Error bars represent the SEM from six animals. ND: not detected.

Figure 5D is a bar graph depicting the influenza viral titer at 3 days after infection in mice treated with 5'ppp-SEQ ID NO: 1 one day prior to and on the day of infection with 500 PFU of influenza; one day prior to, on the day of, and one day following the day of infection with 5'ppp-SEQ ID NO: 1; and mice infected with 500 PFU of influenza but otherwise untreated (NT). Viral titer was determined by plaque assay. Error bars represent the SEM from five different animals.

Figure 5E is a bar graph depicting the influenza viral titer in mice infected with 50 PFU of influenza on day 0 and treated with 5'ppp-SEQ ID NO: 1 on day -1 and day 0 (prophylactic), or on day 1 and day 2 (therapeutic). Lung viral titers were determined on Day 3. Error bars represent the standard error of the mean from five animals.

Figure 5F is a bar graph depicting the results of an ELISA assay for serum IFNβ in wild type, TLR3^{-/-}, and MAVS^{-/-} mice as indicated. All mice were treated with 5'ppp-SEQ

ID NO: 1. IFN β was quantified by ELISA 6 hours. Error bars represent the standard error of the mean from three animals.

Figure 5G is a bar graph depicting the results of wild type and MAVS^{-/-} mice treated with 5'ppp-SEQ ID NO: 1 as indicated and infected with influenza at 500 PFU. Lungs were collected and homogenized on Day 1 and lung viral titers were determined by plaque assay. Error bars represent the standard error of the mean from four different animals.

Figure 5H is a line plot showing survival of IFN α/β R^{-/-} mice treated with 5'ppp-SEQ ID NO: 1 as indicated and infected with influenza at 100 PFU. Survival was monitored for 18 days.

Figure 5I is a bar graph depicting the results of an ELISA assay for serum IFN β in mice treated with 5'ppp-SEQ ID NO: 1 and non-treated (NT) mice. Serum was collected 6 hours after treatment. Error bars represent the SEM from three animals.

Figures 6A-6C demonstrate that 5'ppp-SEQ ID NO: 1 treatment controls influenza-mediated pneumonia.

Figure 6A is an image of representative lung samples from the following groups: In the far left panels animals were treated with neither 5'ppp-SEQ ID NO: 1 nor infected with influenza. In the panels second from left, animals were treated with 5'ppp-SEQ ID NO: 1, but not infected with influenza. In the panels second from right, animals were infected with influenza but not treated with 5'ppp-SEQ ID NO: 1. In the panels on the right, animals were infected with influenza and treated with 5'ppp-SEQ ID NO: 1. Lungs were collected on day 3 and day 8 post-infection and stained with hematoxylin and eosin (H&E). The images in Figure 9A highlight inflammation and tissue damage.

Figure 6B is an image of representative lung samples of influenza infected animals either treated with 5'ppp-SEQ ID NO: 1 (top panel) or untreated (bottom panel) highlighting the extent of pneumonia.

Figure 6C is a bar graph summarizing inflammation, tissue damage and surface area affected by pneumonia of the groups described in the legend for Figure 9A as

scored by a veterinary pathologist. Grade 1 = nil; Grade 2 = modest, rare; Grade 3 = moderate, frequent; Grade 4 = severe, extensive.

Figure 8A (left panel) is a bar graph depicting the VSV virus titer from the supernatants from the experiment described in Fig. 6A was determined by standard plaque assay. The right panel is an image of an immunoblot probed with antibodies specific for VSV proteins.

Figure 8B is a set of two bar graphs depicting the dengue virus titer from supernatants described in Figure 6A determined by plaque assay (left panel) and the virus titer from the supernatants using primers specific for Dengue RNA (SEQ ID NO: 29 and SEQ ID NO: 30.)

Figure 9A is a set of four bar graphs depicting IFN α and IFN β protein expression in the serum and lung homogenates of mice treated with 25 μ g of 5'ppp-SEQ ID NO: 1 in complex with In vivo Jet-PEI™. Protein expression was determined by ELISA at the indicated time post treatment. Error bars represent the standard error of the mean from three animals.

Figure 9B is a set of four bar graphs depicting RIG-I and IFIT1 RNA expression in spleen and lung homogenates of mice treated with 25 μ g of 5'ppp-SEQ ID NO: 1 in complex with In vivo Jet-PEI™. RNA expression was determined by RT-PCR at the indicated time post treatment. Error bars represent the standard error of the mean from three animals.

Figure 9C is a set of three bar graphs depicting the indicated cellular populations in lung homogenates of mice treated with 25 μ g of 5'ppp-SEQ ID NO: 1 in complex with In vivo Jet-PEI™. Lungs were minced and digested with collagenase IV and DNase I for 30 minutes, mixed for 15 minutes, and then filtered through a 70 μ M nylon filter. Cell types were analyzed by flow cytometry and the values given relative to CD45⁺ leukocytes. Error bars represent the standard error of the mean from four animals.

Figure 9D is a set of four bar graphs depicting CXCL10 and IRF7 RNA expression in spleen (left) and lung (right) homogenates of mice treated with 25 μ g of 5'ppp-SEQ ID

NO: 1 in complex with In vivo Jet-PEI™. RNA expression was determined by RT-PCR at the indicated time post treatment. Error bars represent the standard error of the mean from three animals.

Figure 10A is a set of six flow cytometry plots showing infection of A549 cells with Dengue Virus (DENV) with and without 5'ppp-SEQ ID NO: 1.

Figure 10B is a bar graph summarizing flow cytometry data of infection of A549 cells in the presence of the indicated concentration of 5'ppp-SEQ ID NO: 1 or a negative control RNA.

For both Figures 10A and 10B, A549 cells were pretreated with various concentrations of 5'ppp-SEQ ID NO: 1 (0.01 to 10 ng/ml) or control (Ctrl) RNA lacking the 5'ppp at the same concentrations for 24 h prior to DENV challenge. The percentage of DENV-infected cells was determined by intracellular staining (ICS) of DENV E protein expression using flow cytometry. Data are from two independent experiments performed in triplicate and represent the means $\pm$ SEM. *, $P < 0.05$. FSC, forward scatter.

Figure 10C is a bar graph showing DENV RNA expression in DENV infected cells according to the indicated conditions.

Figure 10D is a bar graph showing viral titer and image of a Western blot showing DENV protein expression in DENV infected cells according to the indicated conditions.

For Figures 10C and 10D, A549 cells were pretreated with 5'ppp-SEQ ID NO: 1 (1 ng/ml) for 24 h prior to DENV challenge (MOI, 0.1). DENV RNA level (Figure 10C), viral titers (Figure 10D), and DENV E protein expression level (Figure 10D) were determined by RT-qPCR, plaque assay, and Western blotting, respectively. Error bars represent SEM from three independent samples. *, $P < 0.05$. One representative DENV E protein Western blot out of three independent triplicates is shown.

Figure 10E is a bar graph showing DENV E protein expression in A549 cells infected according to the indicated conditions. A549 cells were transfected using Lipofectamine (Lipo.) RNAiMax with increasing concentrations of 5'ppp-SEQ ID NO: 1

and poly(I:C) (0.1 to 1 ng/ml) or treated with the same dsRNA sequences (5,000 ng/ml) in the absence of transfection reagent. Cells were then challenged with DENV (MOI, 1), and the percentage of infected cells was determined by FACS 24 h after infection. Data are the means $\pm$ SEM from two independent experiments performed in triplicate. *, P < 0.05.

Figure 10F is a bar graph showing DENV E protein expression in A549 cells infected according to the indicated conditions.

Figure 10G is a bar graph showing cell viability in A549 cells treated as indicated. The percentage of A549 DENV-infected cells and cell viability were assessed by flow cytometry and determined at 24 h (black bars), 48 h (gray bars), and 72 h (white bars) after DENV challenge (MOI, 0.01). Cells were pretreated with 5'ppp-SEQ ID NO: 1 (1 ng/ml) for 24 h before DENV challenge. Data are the means $\pm$ SEM from a representative experiment performed in triplicate. *, P < 0.05.

Figure 11A is a bar graph of DENV E protein expression in A549 cells treated according to the indicated conditions. A549 cells were treated with 5'ppp-SEQ ID NO: 1 (1 ng/ml) 4 h (black bars) or 8 h (gray bars) following DENV challenge (MOI, 0.01). The percentage of DENV-infected cells was determined by intracellular staining (ICS) of DENV E protein expression using flow cytometry at 48 h after infection. Data represent the means $\pm$ SEM from a representative experiment performed in triplicate. *, P < 0.05.

Figure 11B is a bar graph of DENV RNA expression in A549 cells treated according to the indicated conditions. DENV RNA levels were determined by RT-qPCR (48 h after infection) on A549 cells treated with 5'pppRNA (1 ng/ml) 4 h (black bars) and 8 h (gray bars) after infection. *, P < 0.05.

Figure 11C is a set of flow cytometry plots indicating the viability of A549 cells treated according to the indicated conditions.

Figure 11D is a bar graph summarizing the flow cytometry data in Figure 11C

Cell viability of A549 cells was measured by flow cytometry 24 h (black bars) and 48 h (gray bars) after infection. Cells were treated with 5'ppp-SEQ ID NO: 1 4 h after

DENV infection. Data are the means $\pm$ SEM from a representative experiment performed in triplicate.

Figure 11E is an image of a western blot indicating expression of the indicated proteins in A549 cells treated according to the indicated conditions. A549 cells were challenged with DENV (MOI, 0.1) for 4 h and transfected with 5'ppp-SEQ ID NO: 1 (0.1 to 10 ng/ml) and incubated for an additional 20 h. Whole-cell extracts (WCEs) were prepared and subjected to immunoblot analysis 24 h postinfection. Data are from one representative experiment.

Figure 11F is a set of four bar graphs indicating expression of the indicated genes in A549 cells treated according to the indicated conditions. A549 cells were infected with DENV at different MOI and were transfected with 5'ppp-SEQ ID NO: 1 (1 ng/ml) 4 h after infection. The expression level of genes was determined by RT-qPCR 24 h after DENV challenge. Data are the means $\pm$ SEM from a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 12A is an image of a western blot indicating the expression of the indicated proteins in A549 cells treated according to the indicated conditions. A549 cells were transfected with control or RIG-I siRNA (10 or 30 pmol), and 48 h later they were treated with 5'ppp-SEQ ID NO: 1 (10 ng/ml) for 24 h. Expression of IFIT1, RIG-I, and β -actin was evaluated by Western blotting. RIG-I knockdown and impairment of the 5'ppp-SEQ ID NO: 1-induced immune response is representative of at least 3 independent experiments.

Figure 12B is a set of four bar graphs indicating the expression of the indicated genes in A549 cells treated according to the indicated conditions. A549 cells were transfected with control siRNA or RIG-I siRNA (30 pmol), and 48 h later they were treated with 5'ppp-SEQ ID NO: 1 (10 ng/ml) for 24 hours. mRNA expression level of IFN- α , IFN- β , TNF- α , and IL-29 was evaluated by RT-qPCR. Data are from a representative experiment performed in triplicate and show the means $\pm$ SEM. *, $P < 0.05$.

Figure 12C is a bar graph of indicating the expression of DENV E protein in A549 cells treated according to the indicated conditions. A549 cells were transfected with control (black bars), RIG-I (gray bars), or a combination of TLR3/MDA5 (white bars) siRNA (30 pmol each), and 48 h later they were treated with 5'ppp-SEQ ID NO: 1 (10 ng/ml) or poly(I:C) (1 ng/ml). Cells were then infected with DENV (MOI, 0.5), and at 24 h p.i. the percentage of infected cells was assessed by intracellular staining of DENV E protein using flow cytometry. Data are from a representative experiment performed in triplicate and show the means $\pm$ SEM. *, $P < 0.05$.

Figure 12D is a bar graph indicating the expression of DENV E protein in A549 cells treated according to the indicated conditions.

Figure 12E is a bar graph indicating the expression of DENV E protein in A549 cells treated according to the indicated conditions.

For both Figures 12D and 12E: A549 cells were treated with 5'ppp-SEQ ID NO: 1 (0.1 to 10 ng/ml) for 24 h 2 days after transfection with 30 pmol of control (black bars), RIG-I (gray bars), or STING (white bars) siRNA (Figure 12D) or with 30 pmol of control (black bars) or MAVS (gray bars) siRNA (Figure 12E). Cells were then challenged with DENV (MOI, 0.1) for 24 h. The percentage of DENV-infected cells was determined by intracellular staining of DENV E protein and flow cytometry 24 h after infection. Data are the means $\pm$ SEM from a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 12F is a bar graph indicating the expression of DENV E protein in A549 cells treated according to the indicated conditions. TBK1^{+/+} (black bars) and TBK1^{-/-} (gray bars) MEF cells were treated with 10 ng/ml of 5'ppp-SEQ ID NO: 1 24 h before DENV challenge at an MOI of 5. The percentage of DENV-infected cells was evaluated by flow cytometry. Data are the means $\pm$ SEM of a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 13A is a set of three bar graphs indicating the expression of the indicated genes in A549 treated according to the indicated conditions. A549 cells were transfected with control, IFN- α /IFN- β chain (IFNAR1), IFN- α /IFN- β chain (IFNAR2), or IL-28R siRNA, and

48 h later mRNA levels of IFNAR1, IFNAR2, and IL-28R were evaluated by RT-qPCR. Data are from a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 13B is an image of a Western blot showing the expression of the indicated proteins in A549 cells treated according to the indicated conditions. A549 cells were transfected with the control siRNA, IFN- α / β R or IL-28R siRNA, or a combination of both. After 48 h, cells were treated with 5'ppp-SEQ ID NO: 1 (10 ng/ml) or IFN- α 2b (100 UI/ml) for 24 h. Expression of IFIT1, RIG-I, and β -actin was evaluated by Western blotting. The evaluation of 5'ppp-SEQ ID NO: 1-induced immune response by Western blotting in the absence of type I IFN receptor, representative of three independent experiments, and in the absence of type III IFN receptor, representative of one experiment.

Figure 13C is a bar graph indicating the expression of DENV E protein in A549 cells treated according to the indicated conditions. After siRNA knockdown of IFN- α / β R as described for in Figure 13B, cells were treated with increasing concentrations of 5'ppp-SEQ ID NO: 1 (0.1 to 10 ng/ml) and then infected with DENV (MOI, 0.1). The percentage of DENV-infected cells was evaluated by flow cytometry. Data are the means $\pm$ SEM of a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 13D is an image of a Western Blot showing the expression of the indicated proteins in A549 cells treated according to the indicated conditions. A549 cells were transfected with control and STAT1 siRNA, and 48 h later they were treated with 5'ppp-SEQ ID NO: 1 (0.01 to 1 ng/ml) for 24 h. Expression of STAT1, IFIT1, and β -actin was evaluated by Western blotting. The induction of 5'ppp-SEQ ID NO: 1-induced immune response in the absence of STAT is representative of two independent experiments.

Figure 13E is a bar graph showing the expression of DENV E protein in A549 cells treated according to the indicated conditions. A549 cells were transfected with control or STAT1 siRNA and incubated for 48 h. Cells were treated with increasing concentrations of 5'ppp-SEQ ID NO: 1 (0.1 to 10 ng/ml) and then infected with DENV (MOI, 0.1). The percentage of DENV-infected cells was evaluated by flow cytometry.

Data are the means $\pm$ SEM from a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 13F is an image of a Western blot showing the expression of the indicated proteins in A549 cells treated according to the indicated conditions. A549 cells were transfected with control, IRF1, IRF3, or IRF7 siRNA for 48 h, and the protein expression level of these transcription factors was evaluated by Western blotting. This panel is representative of one experiment.

Figure 13G is a bar graph showing the expression of DENV E protein in A549 cells treated according to the indicated conditions. A549 cells were transfected with control IRF1, IRF3, or IRF7 and then treated as described for panel E. The percentage of DENV-infected cells was evaluated by flow cytometry. Data are the means $\pm$ SEM from a representative experiment performed in triplicate. *, $P < 0.05$.

Figure 14A is a set of eight flow cytometry histograms showing the expression of DENV E protein in A549 cells treated according to the indicated conditions. Negatively selected monocytes were challenged with DENV (MOI, 20) in the presence or absence of the enhancing antibody 4G2 (0.5 $\mu\text{g/ml}$) for 4 h. They were subsequently transfected with 5'ppp-SEQ ID NO: 1 (100 ng/ml) using Lyovec and incubated for 20 h. An IgG2a antibody (0.5 $\mu\text{g/ml}$) served as a negative control. The percentage of DENV-infected cells was determined by flow cytometry 24 h after infection.

Figure 14B is a bar graph showing the expression of DENV E protein in A549 cells treated according to the indicated conditions. CD14⁺ MDDCs were challenged with DENV (MOI, 10) for 4 h, followed by transfection with 5'ppp-SEQ ID NO: 1 (100 ng/ml) and incubation for an additional 20 h. Data represent the means $\pm$ SEM of an experiment performed in triplicate. *, $P < 0.05$.

Figure 14C is a bar graph showing the percentage of viable A549 cells treated according to the indicated conditions. Cell viability was assessed by flow cytometry on CD14⁺ MDDC and determined 24 h after 5'ppp-SEQ ID NO: 1 treatment (10 to 500

ng/ml) in the presence of Lyovec. Data are the means $\pm$ SEM of a representative experiment performed in triplicate.

Figure 14D is an image of a Western blot showing the expression of the indicated proteins in A549 cells treated according to the indicated conditions. CD14⁺ MDDCs were challenged with DENV (MOI, 10) for 4 h and then were treated with 5'ppp-SEQ ID NO: 1 (100 ng/ml) for an additional 20 h. WCEs were resolved by SDS-PAGE and analyzed by immunoblotting for phospho-IRF3, IRF3, phospho-STAT1, STAT1, IFIT1, RIG-I, STING, and β -actin. Results are from one representative experiment that was repeated once.

Figure 15A is a plot showing reporter gene expression in MRC-5 cells infected with CHIKV LS3-GFP and treated according to the indicated conditions. MRC-5 cells were treated with 0.015 to 4 ng/ml of control RNA or 5'ppp-SEQ ID NO: 1 from 1 h prior to infection to 24 h postinfection with CHIKV LS3-GFP (MOI, 0.1). At 24 h p.i., cells were fixed and EGFP reporter gene expression was quantified. *, $P < 0.05$. cntrl, control.

Figure 15B is a plot showing cell viability in MRC-5 cells infected with CHIKV LS3-GFP and treated according to the indicated conditions. To assess potential cytotoxicity, MRC-5 cell viability was measured 24 h posttransfection of 5'ppp-SEQ ID NO: 1 or control RNA lacking the 5' triphosphate. Data are represented as the means $\pm$ SEM from a representative experiment performed in quadruplicate.

Figure 15C is an image of a Northern blot showing the intracellular accumulation of CHIKV positive and negative strand RNA in MRC-5 cells treated according to the indicated conditions. The intracellular accumulation of CHIKV positive- and negative-strand RNA was determined by in-gel hybridization of RNA isolated from MRC-5 cells that were treated with 5'ppp-SEQ ID NO: 1 (0.1 to 10 ng/ml) 1 h prior to infection (MOI, 0.1).

Figure 15D is an image of a Western blot showing the expression of the indicated CHIKV proteins in MRC-5 cells infected with CHIKV and treated according to the indicated conditions. CHIKV E2, E3E2, and nsP1 protein expression was assessed by Western blotting of lysates of MRC-5 cells that were treated with various concentrations

of control RNA or 5'ppp-SEQ ID NO: 1 1 h prior to infection with CHIKV. Data are representative of at least two independent experiments.

Figure 15E is a bar graph showing the CHIKV titer in MRC-5 cells infected with CHIKV and treated according to the indicated conditions as assessed by plaque assay.

Figure 15F is a bar graph of reporter gene expression in MRC-5 cells infected with CHIKV LS3-GFP, transfected with the indicated siRNA and treated according to the indicated conditions. siRNA transfected MRC-5 cells were either left untreated or were transfected with 5'ppp-SEQ ID NO: 1, after which they were infected with CHIKV LS3-GFP (MOI, 0.1). CHIKV-driven EGFP reporter gene expression was measured at 24 h p.i. and was normalized to the expression level in CHIKV-infected cells that had been transfected with a nontargeting scrambled siRNA (scr). *, $P < 0.05$.

Figure 15G is a set of three images of Western blots showing the expression of the indicated proteins in MRC-5 cells infected with CHIKV and treated according to the indicated conditions. MRC-5 cells were transfected with 10 pmol of scrambled siRNA (siScr) or siRNA targeting RIG-I, STAT1, or STING 48 h prior to treatment with 1 ng/ml of 5'ppp-SEQ ID NO: 1. Expression levels of RIG-I, STAT1, STING, and IFIT1 were monitored by Western blotting. Cyclophilin A or B was used as a loading control. Data are representative of at least two independent experiments.

For all of Figures 16A, 16B, and 16C, MRC-5 cells were infected with CHIKV LS3-GFP at an MOI of 0.1, and at the indicated time points postinfection they were transfected with 1 ng/ml 5'ppp-SEQ ID NO: 1, or control RNA.

Figure 16A is a bar graph of reporter gene expression in MRC-5 cells described above treated according to the indicated conditions. Cells were fixed at 24 h p.i., and EGFP reporter gene expression was quantified and normalized to that in untreated cells. *, $P < 0.05$.

Figure 16B is a bar graph of CHIKV virus titer in the MRC-5 cells described above. CHIKV progeny titers 24 h p.i. and after 5'ppp-SEQ ID NO: 1 or control RNA treatment were determined by plaque assay.

Figure 16C is a set of 24 images from Western blots from the cells described above showing the expression of the indicated proteins in cells treated according to the indicated conditions. MRC-5 cells were transfected with 0.1, 1, or 10 ng/ml 5'ppp-SEQ ID NO: 1 or control RNA 1 h prior to infection with CHIKV LS3-GFP (MOI, 0.1). At 24 h p.i., cell lysates were prepared and STAT1, RIG-I, and IFIT-I protein levels were determined by Western blotting. Actin or the transferrin receptor were used as loading controls. Data are representative of at least two independent experiments.

SEQUENCE LISTING

SEQ ID NO: 1 is an oligoribonucleotide derived from the 5' UTR and 3' UTR of vesicular stomatitis virus (VSV).

SEQ ID NO: 2 is the sequence of DNA template encoding the oligoribonucleotide of SEQ ID NO: 1.

SEQ ID NO: 3 is a forward primer for the detection of IFNB1 expression by RT-PCR.

SEQ ID NO: 4 is a reverse primer for the detection of IFNB1 expression by RT-PCR.

SEQ ID NO: 5 is a forward primer for the detection of IL29 expression by RT-PCR.

SEQ ID NO: 6 is a reverse primer for the detection of IL29 expression by RT-PCR.

SEQ ID NO: 7 is a forward primer for the detection of IRF7 expression by RT-PCR.

SEQ ID NO: 8 is a reverse primer for the detection of IRF7 expression by RT-PCR.

SEQ ID NO: 9 is a forward primer for the detection of CCL5 expression by RT-PCR.

SEQ ID NO: 10 is a reverse primer for the detection of CCL5 expression by RT-PCR.

SEQ ID NO: 11 is a forward primer for the detection of CXCL10 expression by RT-PCR.

SEQ ID NO: 12 is a reverse primer for the detection of CXCL10 expression by RT-PCR.

SEQ ID NO: 13 is a forward primer for the detection of IL6 expression by RT-PCR.

SEQ ID NO: 14 is a reverse primer for the detection of IL6 expression by RT-PCR.

SEQ ID NO: 15 is a forward primer for the detection of ISG15 expression by RT-PCR.

SEQ ID NO: 16 is a reverse primer for the detection of ISG15 expression by RT-PCR.

SEQ ID NO: 17 is a forward primer for the detection of ISG56 expression by RT-PCR.

SEQ ID NO: 18 is a reverse primer for the detection of ISG56 expression by RT-PCR.

SEQ ID NO: 19 is a forward primer for the detection of RIG-I expression by RT-PCR.

SEQ ID NO: 20 is a reverse primer for the detection of RIG-I expression by RT-PCR.

SEQ ID NO: 21 is a forward primer for the detection of Viperine expression by RT-PCR.

SEQ ID NO: 22 is a reverse primer for the detection of Viperine expression by RT-PCR.

SEQ ID NO: 23 is a forward primer for the detection of OASL expression by RT-PCR.

SEQ ID NO: 24 is a reverse primer for the detection of OASL expression by RT-PCR.

SEQ ID NO: 25 is a forward primer for the detection of NOXA expression by RT-PCR.

SEQ ID NO: 26 is a reverse primer for the detection of NOXA expression by RT-PCR.

SEQ ID NO: 27 is a forward primer for the detection of GADPH expression by RT-PCR.

SEQ ID NO: 28 is a reverse primer for the detection of GADPH expression by RT-PCR.

SEQ ID NO: 29 is a forward primer for the detection of Dengue virus RNA expression by RT-PCR.

SEQ ID NO: 30 is a reverse primer for the detection of Dengue virus RNA expression by RT-PCR.

SEQ ID NO: 31 is a forward primer for the detection of DENV2

SEQ ID NO: 32 is a reverse primer for the detection of DENV2.

SEQ ID NO: 33 is a forward primer for the detection of GADPH.

SEQ ID NO: 34 is a reverse primer for the detection of GADPH.

SEQ ID NO: 35 is a forward primer for the detection of IFNA2.

SEQ ID NO: 36 is a reverse primer for the detection of IFNA2.

SEQ ID NO: 37 is a forward primer for the detection of IFNAR1.

SEQ ID NO: 38 is a reverse primer for the detection of IFNAR1.

SEQ ID NO: 39 is a forward primer for the detection of IFNAR2.

SEQ ID NO: 40 is a reverse primer for the detection of IFNAR2.

SEQ ID NO: 41 is a forward primer for the detection of IFNB1

SEQ ID NO: 42 is a reverse primer for the detection of IFNB1

SEQ ID NO: 43 is a forward primer for the detection of ILA.

SEQ ID NO: 44 is a reverse primer for the detection of ILA.

SEQ ID NO: 45 is a forward primer for the detection of IL-6.

SEQ ID NO: 46 is a reverse primer for the detection of IL-6.

SEQ ID NO: 47 is a forward primer for the detection of IL28RA.

SEQ ID NO: 48 is a reverse primer for the detection of IL28RA.

SEQ ID NO: 49 is a forward primer for the detection of IL-29.

SEQ ID NO: 50 is a reverse primer for the detection of IL-29.

SEQ ID NO: 51 is a forward primer for the detection of TNFA

SEQ ID NO: 52 is a reverse primer for the detection of TNFA.

SEQ ID NO: 53 is the CHIKVhyb4 probe.

SEQ ID NO: 54 is the CHIKVhyb2 probe.

DETAILED DESCRIPTION

Disclosed herein is a oligoribonucleotide of SEQ ID NO: 1 comprising a triphosphate group on the 5' end (5'ppp-SEQ ID NO: 1), pharmaceutical compositions comprising the oligoribonucleotide, and methods of using the oligoribonucleotide to treat viral infections.

A DNA plasmid may be used to generate an oligoribonucleotide of SEQ ID NO: 1. Such a plasmid may include SEQ ID NO: 2. The oligoribonucleotide can be transcribed as an RNA molecule that automatically folds into duplexes with hairpin loops. Typically, a transcriptional unit or cassette will contain an RNA transcript promoter sequence, such as a T7 promoter operably linked to SEQ ID NO: 2 for transcription of 5'ppp-SEQ ID NO: 1.

Methods of isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (see, e.g., Gubler and Hoffman, *Gene* **25**, 263-269 (1983); Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, (2001)) as are PCR methods (see, U.S. Pat. Nos. 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications*, Innis et al, eds, (1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook and Russell (2001) *supra*; Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel et al., eds., 1994).

An oligoribonucleotide may be chemically synthesized. Synthesis of the single-stranded nucleic acid makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems

synthesizer using a 0.2 micromolar scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides. Alternatively, syntheses at the 0.2 micromolar scale can be performed on a 96-well plate synthesizer from Protogene. However, a larger or smaller scale of synthesis is encompassed by the invention, including any method of synthesis now known or yet to be disclosed. Suitable reagents for synthesis of the siRNA single-stranded molecules, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

An oligoribonucleotide can be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous fragment or strand separated by a linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form an RNA duplex. The linker may be any linker, including a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of RNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, the oligoribonucleotide can be assembled from two distinct single-stranded molecules, wherein one strand includes the sense strand and the other includes the antisense strand of the RNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. Either the sense or the antisense strand may contain additional nucleotides that are not complementary to one another and do not form a double stranded RNA molecule. In certain other instances, the oligoribonucleotide can be synthesized as a single continuous fragment, where the self-complementary sense and antisense regions hybridize to form an RNA duplex having a hairpin or panhandle secondary structure.

An oligoribonucleotide may comprise a duplex having two complementary strands that form a double-stranded region with least one modified nucleotide in the double-stranded region. The modified nucleotide may be on one strand or both. If the modified nucleotide is present on both strands, it may be in the same or different

positions on each strand. Examples of modified nucleotides suitable for use in the present invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a conformation such as those described in, for example in Sanger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in oligoribonucleotides. Other modified nucleotides include, without limitation: locked nucleic acid (LNA) nucleotides, G-clamp nucleotides, or nucleotide base analogs. LNA nucleotides include but need not be limited to 2'-O, 4'-C-methylene-(D-ribofuranosyl)nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2Cl) nucleotides, and 2'-azido nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (Lin *et al*, *J Am Chem Soc*, **120**, 8531-8532 (1998)). Nucleotide base analogs include for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine,azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (Loakes, *Nucl Acids Res*, **29**, 2437-2447 (2001)).

An oligoribonucleotide may comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of classes of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β -D-erythrofuransyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α -nucleotides, modified base nucleotides, threo pentofuransyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted

deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3 aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5' phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (see, e.g., U.S. Pat. No. 5,998,203; Beaucage *et al*, *Tetrahedron* 49, 1925 (1993)). Non-limiting examples of phosphate backbone modifications (i.e., resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (see, e.g., Hunziker *et al*, *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al*, *Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the oligoribonucleotide.

The sense and/or antisense strand of an oligoribonucleotide may comprise a 3'-terminal overhang having 1 to 4 or more 2'-deoxyribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into the modified oligoribonucleotides of the present invention are described, e.g., in UK Patent No. GB 2,397,818 B and U.S. Patent Publication Nos. 20040192626 and 20050282188.

An oligoribonucleotide may comprise one or more non-nucleotides in one or both strands of the siRNA. A non-nucleotide may be any subunit, functional group, or other molecular entity capable of being incorporated into a nucleic acid chain in the place of one or more nucleotide units that is not or does not comprise a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine, such as a sugar or phosphate.

Chemical modification of the oligoribonucleotide may also comprise attaching a conjugate to the oligoribonucleotide molecule. The conjugate can be attached at the 5'- and/or the 3'-end of the sense and/or the antisense strand of the oligoribonucleotide via a covalent attachment such as a nucleic acid or non-nucleic acid linker. The conjugate can also be attached to the oligoribonucleotide through a carbamate group or other linking group (see, e.g., U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). A conjugate may be added to the oligoribonucleotide for any of a number of purposes. For example, the conjugate may be a molecular entity that facilitates the delivery of the oligoribonucleotide into a cell or the conjugate a molecule that comprises a drug or label.

Examples of conjugate molecules suitable for attachment to the disclosed oligoribonucleotides include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (e.g., folic acid, folate analogs and derivatives thereof), sugars (e.g., galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, etc.), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (see, e.g., U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Pat. No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidinium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples of conjugate molecules include a hydrophobic group, a membrane active compound, a cell penetrating compound, a cell targeting signal, an interaction modifier, or a steric

stabilizer as described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739.

The type of conjugate used and the extent of conjugation to the oligoribonucleotide can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the oligoribonucleotide while retaining activity. As such, one skilled in the art can screen oligoribonucleotides having various conjugates attached thereto to identify oligonucleotide conjugates having improved properties using any of a variety of well-known in vitro cell culture or in vivo animal models.

An oligoribonucleotide may be incorporated into a pharmaceutically acceptable carrier or transfection reagent containing the oligoribonucleotides described herein. The carrier system may be a lipid-based carrier system such as a stabilized nucleic acid-lipid particle (e.g., SNALP or SPLP), cationic lipid or liposome nucleic acid complexes (i.e., lipoplexes), a liposome, a micelle, a virosome, or a mixture thereof. In other embodiments, the carrier system is a polymer-based carrier system such as a cationic polymer-nucleic acid complex (i.e., polyplex). In additional embodiments, the carrier system is a cyclodextrin-based carrier system such as a cyclodextrin polymer-nucleic acid complex (see US Patent Application Publication 20070218122). In further embodiments, the carrier system is a protein-based carrier system such as a cationic peptide-nucleic acid complex. An oligoribonucleotide molecule may also be delivered as naked RNA.

A pharmaceutical composition may be any chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject. A pharmaceutical composition can include a therapeutic agent, a diagnostic agent or a pharmaceutical agent. A therapeutic or pharmaceutical agent is one that alone or together with an additional compound induces the desired response (such as inducing a therapeutic or prophylactic effect when administered to a subject). In a particular example, a pharmaceutical agent is an agent that significantly reduces one or more symptoms associated with viral infection. A pharmaceutical

composition may be a member of a group of compounds. Pharmaceutical compositions may be grouped by any characteristic including chemical structure and the molecular target they affect.

A pharmaceutically acceptable carrier (interchangeably termed a vehicle) may be any material or molecular entity that facilitates the administration or other delivery of the pharmaceutical composition. In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle.

A therapeutically effective amount or concentration of a compound such as 5'ppp-SEQ ID NO: 1 may be any amount of a composition that alone, or together with one or more additional therapeutic agents is sufficient to achieve a desired effect in a subject, or in a cell being treated with the agent. The effective amount of the agent will be dependent on several factors, including, but not limited to, the subject or cells being treated and the manner of administration of the therapeutic composition. In one example, a therapeutically effective amount or concentration is one that is sufficient to prevent advancement, delay progression, or to cause regression of a disease, or which is capable of reducing symptoms caused by any disease, including viral infection.

In one example, a desired effect is to reduce or inhibit one or more symptoms associated with viral infection. The one or more symptoms do not have to be completely eliminated for the composition to be effective. For example, a composition can decrease the sign or symptom by a desired amount, for example by at least 20%, at least 50%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%, as compared to the sign or symptom in the absence of the composition.

A therapeutically effective amount of a pharmaceutical composition can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the therapeutically effective amount can depend on the subject

being treated, the severity and type of the condition being treated, and the manner of administration. For example, a therapeutically effective amount of such agent can vary from about 100 µg -10 mg per kg body weight if administered intravenously.

The actual dosages will vary according to factors such as the type of virus to be protected against and the particular status of the subject (for example, the subject's age, size, fitness, extent of symptoms, susceptibility factors, and the like) time and route of administration, other drugs or treatments being administered concurrently, as well as the specific pharmacology of treatments for viral infection for eliciting the desired activity or biological response in the subject. Dosage regimens can be adjusted to provide an optimum prophylactic or therapeutic response.

A therapeutically effective amount is also one in which any toxic or detrimental side effects of the compound and/or other biologically active agent is outweighed in clinical terms by therapeutically beneficial effects. A non-limiting range for a therapeutically effective amount of treatments for viral infection within the methods and formulations of the disclosure is about 0.0001 µg/kg body weight to about 10 mg/kg body weight per dose, such as about 0.0001 µg/kg body weight to about 0.001 µg/kg body weight per dose, about 0.001 µg/kg body weight to about 0.01 µg/kg body weight per dose, about 0.01 µg/kg body weight to about 0.1 µg/kg body weight per dose, about 0.1 µg/kg body weight to about 10 µg/kg body weight per dose, about 1 µg/kg body weight to about 100 µg/kg body weight per dose, about 100 µg/kg body weight to about 500 µg/kg body weight per dose, about 500 µg/kg body weight per dose to about 1000 µg/kg body weight per dose, or about 1.0 mg/kg body weight to about 10 mg/kg body weight per dose.

Dosage can be varied by the attending clinician to maintain a desired concentration. Higher or lower concentrations can be selected based on the mode of delivery, for example, trans-epidermal, rectal, oral, pulmonary, intranasal delivery, intravenous or subcutaneous delivery.

Determination of effective amount is typically based on animal model studies followed up by human clinical trials and is guided by administration protocols that significantly reduce the occurrence or severity of targeted disease symptoms or conditions in the subject. Suitable models in this regard include, for example, murine, rat, porcine, feline, non-human primate, and other accepted animal model subjects known in the art. Alternatively, effective dosages can be determined using in vitro models (for example, viral titer assays or cell culture infection assays). Using such models, only ordinary calculations and adjustments are required to determine an appropriate concentration and dose to administer a therapeutically effective amount of the treatments for viral infection (for example, amounts that are effective to alleviate one or more symptoms of viral infection).

Methods of treating viral infections

Disclosed herein are methods of treating a subject that has or may have a viral infection comprising administering a pharmaceutical composition comprising 5'ppp-SEQ ID NO: 1 to the subject. The subject may be treated therapeutically or prophylactically.

A subject may be any multi-cellular vertebrate organisms, a category that includes human and non-human mammals, such as mice. In some examples a subject is a male. In some examples a subject is a female. Further types of subjects to which the pharmaceutical composition may be properly administered include subjects known to have a viral infection (through, for example, a molecular diagnostic test or clinical diagnosis,) subjects having a predisposition to contracting a viral infection (for example by living in or travelling to a region in which one or more viruses is endemic), or subjects displaying one or more symptoms of having a viral infection.

Administration of a pharmaceutical composition may be any method of providing or give a subject a pharmaceutical composition comprising 5'ppp-SEQ ID NO: 1, by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal,

and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Treating a subject may be any therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop, whether or not the subject has developed symptoms of the disease. Ameliorating, with reference to a disease, pathological condition or symptom refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of relapses of the disease, an improvement in the memory and/or cognitive function of the subject, a qualitative improvement in symptoms observed by a clinician or reported by a patient, or by other parameters well known in the art that are specific to viral infections generally or specific viral infections.

A symptom may be any subjective evidence of disease or of a subject's condition, for example, such evidence as perceived by the subject; a noticeable change in a subject's condition indicative of some bodily or mental state. A sign may be any abnormality indicative of disease, discoverable on examination or assessment of a subject. A sign is generally an objective indication of disease.

The administration of a pharmaceutical composition comprising 5'ppp-SEQ ID NO: 1 can be for either prophylactic or therapeutic purposes. When provided prophylactically, the treatments are provided in advance of any clinical symptom of viral infection. Prophylactic administration serves to prevent or ameliorate any subsequent disease process. When provided therapeutically, the compounds are provided at (or shortly after) the onset of a symptom of disease. For prophylactic and therapeutic purposes, the treatments can be administered to the subject in a single bolus delivery, via continuous delivery (for example, continuous transdermal, mucosal or intravenous delivery) over an extended time period, or in a repeated administration protocol (for

example, by an hourly, daily or weekly, repeated administration protocol). The therapeutically effective dosage of the treatments for viral infection can be provided as repeated doses within a prolonged prophylaxis or treatment regimen that will yield clinically significant results to alleviate one or more symptoms or detectable conditions associated with viral infection.

Suitable methods, materials, and examples used in the practice and/or testing of embodiments of the disclosed invention are described below. Such methods and materials are illustrative only and are not intended to be limiting. Other methods, materials, and examples similar or equivalent to those described herein can be used.

EXAMPLES

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

Example 1 – 5'-ppp-SEQ ID NO: 1 stimulates an antiviral response in lung epithelial A549 cells.

A short RNA oligomer derived from the 5' and 3' UTRs of the negative-strand RNA virus Vesicular Stomatitis Virus (VSV) was generated by in vitro transcription using T7 polymerase, an enzymatic reaction that synthesizes RNA molecules with a 5'ppp terminus (5'-ppp-SEQ ID NO: 1). The predicted panhandle secondary structure of the 5'ppp-SEQ ID NO: 1 is depicted in Fig. 1A. Gel analysis and nuclease sensitivity confirmed the synthesis of a single RNA product of the expected length of 67 nucleotides.

The transfection of 5'ppp-SEQ ID NO: 1 into A549 cells resulted in Ser396 phosphorylation of IRF3 at 8 hours – a hallmark of immediate early activation of the antiviral response (Fig. 1B, see particularly lanes 2 to 6). Induction of apoptosis was also detected following treatment with higher concentrations of 5'-ppp-SEQ ID NO: 1. Furthermore, the pro-apoptotic protein NOXA – a direct transcriptional target of IRF3 –

as well as cleavage products of caspase 3 and PARP were up-regulated in a dose dependent manner upon transfection with 5'ppp-SEQ ID NO: 1.) (See Gobau D *et al*, *Eur J Immunol* **39**, 527-540 (2009), incorporated by reference herein). Optimal induction of antiviral signaling with limited cytotoxicity was achieved at a concentration of 10 ng/ml (about 500 pM) (Fig. 1B; lane 4). The stimulation of immune signaling and apoptosis was dependent on the 5'ppp moiety. A homologous RNA without a 5'ppp terminus did not stimulate immune signaling and apoptosis over a range of RNA concentrations (Fig. 1B, lanes 8 to 12).

To characterize the antiviral response triggered by 5'ppp-SEQ ID NO: 1, the kinetics of downstream RIG-I signaling were measured at different times (0-48 hours) after stimulation of A549 cells (Fig. 1C). IRF3 homodimerization (top panel) and IRF3 phosphorylation at Ser396 (2nd panel) were first detected as early as 2 hours post treatment with 5'ppp-SEQ ID NO: 1 and remained until 24 hours post treatment. Expression of endogenous IRF7 was detected later than that of IRF3 (4th panel vs. 3rd panel). I κ B α phosphorylation was detected as early as 2 hours post-treatment and was sustained throughout the time course (6th panel). IRF3, IRF7 and NF- κ B are required for optimal induction of the IFN β promoter.

Tyr701 phosphorylation of STAT1, indicative of JAK-STAT signaling was first detected at 4 hours post treatment with 5'ppp-SEQ ID NO: 1 (9th panel). Tyr 701 phosphorylation was still detected at 24 hours post treatment (10th panel). IFIT1 and RIG-I were both upregulated 4 hours following treatment (11th and 12th panel) while STAT1 and IRF7 (4th and 10th panel) were upregulated 6 hours and 8 hours after treatment (respectively). IFN β was detectable in cell culture supernatant as early as 6 hours after treatment with a peak concentration of 4000pg/ml between 12 and 24 hours after treatment (Fig. 1D, top panel). IFN α was first detected at 12 hours after treatment and remained at a concentration of 400 pg/ml throughout the rest of the time course (Figure 1D, bottom panel).

Example 2 – 5'-ppp-SEQ ID NO: 1 induction of the antiviral response requires an intact RIG-I pathway.

To address whether 5'ppp-SEQ ID NO: 1 exclusively activates RIG-I, wild type mouse embryonic fibroblasts (wtMEF) and RIG-I^{-/-} MEF were co-transfected with 5'ppp-SEQ ID NO: 1 and type 1 IFN reporter constructs to measure promoter activity. 5'ppp-SEQ ID NO: 1 activated the IFN β promoter 60-fold and the IFN α promoter 450-fold in wtMEF. However, 5'ppp-SEQ ID NO: 1 activated neither promoter in RIG-I^{-/-} MEF. A constitutively active RIG-I mutant (described in Yoneyama M *et al*, *Nat Immunol* **5**, 730-737 (2004); incorporated by reference herein) was used in a similar experiment (Figure 2A). Induction of the IFN response by 5'ppp-SEQ ID NO: 1 was dependent on an intact RIG-I signaling pathway because IFN β promoter activity was unchanged by treatment with 5'ppp-SEQ ID NO: 1 in Mda5^{-/-}, TLR3^{-/-}, or TLR7^{-/-} MEFs (Figure 2B). In A549 cells treated with 5'ppp-SEQ ID NO: 1, in which RIG-I expression was silenced using siRNA, IRF3 and STAT1 phosphorylation as well as IFIT1 and RIG-I upregulation were inhibited when compared to control cells treated with an irrelevant siRNA. Transient transfection of irrelevant and specific siRNA did not activate immune signaling (Figures 2C and 2D).

Example 3 - 5'-ppp-SEQ ID NO: 1 acts as a broad-spectrum antiviral agent.

A549 cells were treated with 5'ppp-SEQ ID NO: 1 and 24 hours later were infected with VSV, Dengue (DENV), or Vaccinia viruses. All viruses were able to infect untreated cells (60%, 20% and 80%, respectively as assessed by flow cytometry). In cells pretreated with 5'ppp-SEQ ID NO: 1, VSV and DENV infectivity was less than 0.5%, while infection with vaccinia was about 10% (Fig. 3A). Release of infectious VSV and DENV was blocked by treatment with 5'ppp-SEQ ID NO: 1. VSV infection produced 1.7×10^9 pfu/ml in untreated cells. No plaque forming units were detectable in cells pretreated with 5'ppp-SEQ ID NO: 1. Similarly, DENV infection produced 4.3×10^6 pfu/ml in untreated cells while no plaque forming units were detectable in cells pretreated with 5'ppp-SEQ ID NO: 1. In primary human CD14⁺ monocytes, DENV infection was 53.7%, compared to

2.6% infection in CD14⁺ monocytes pretreated with 5'ppp-SEQ ID NO: 1. In CD14⁻ monocytes, DENV infectivity was 3% in untreated cells, but in 0.4% in cells pretreated with 5'ppp-SEQ ID NO: 1 (Fig. 3B).

In another experiment, primary CD14⁺ monocytes from three human subjects were infected with DENV and treated with 5'ppp-SEQ ID NO: 1 alone, transfection reagent alone or 5'ppp-SEQ ID NO: 1 with transfection agent. 5'ppp-SEQ ID NO: 1 alone or transfection agent alone resulted in an infection rate of about 30%, while cells treated with both transfection agent and 5'ppp-SEQ ID NO: 1 had an infection rate of about 0.5% (Fig 3C).

To evaluate the antiviral effect of 5'ppp-SEQ ID NO: 1 against HIV infection, activated CD4⁺ T cells were pre-treated with supernatant isolated from 5'ppp-SEQ ID NO: 1 treated monocytes and then infected with HIV-GFP (MOI = 0.1). In the absence of treatment with the supernatant, 24% of the activated CD4⁺ T cells were infected by HIV. In cells treated with the supernatant, 11% of the cells were infected (Fig 3D).

5'ppp-SEQ ID NO: 1 also has an antiviral effect against HCV in the hepatocellular carcinoma cell line Huh7. Expression of HCV NS3 was inhibited by 5'ppp-SEQ ID NO: 1 treatment (Fig. 3E; lane 4 vs. 2 and 6). The antiviral effect was dependent on RIG-I. Huh7.5 cells have a mutant inactive RIG-I. These cells did not upregulate IFIT1 upon 5'ppp-SEQ ID NO: 1 treatment (Fig. 3E; lane 9). Furthermore, NS3 expression Huh7.5 cells was comparable to that of untreated HCV-infected cells (Fig. 3E; lane 10 vs. 8 and 12).

Example 4 – 5'-ppp-SEQ ID NO: 1 inhibits H1N1 Influenza infection in vitro

A549 cells were pre-treated with 5'ppp-SEQ ID NO: 1 for 24 hours and then infected with H1N1 A/PR/8/34 Influenza virus at increasing MOI ranging from 0.02 to 2. Influenza replication was monitored by immunoblot analysis of NS1 protein expression (Fig. 4A) and plaque assay (Fig.4B). Viral replication was blocked by 5'ppp-SEQ ID NO: 1 pre-treatment as demonstrated by a complete loss of NS1 expression and a 40-fold decrease in viral titer at an MOI of 2. In another experiment, A549 cells were pre-

treated with decreasing concentrations of 5'ppp-SEQ ID NO: 1 (10 to 0.1 ng/ml) prior to influenza virus challenge at 0.2 MOI. 5'ppp-SEQ ID NO: 1 significantly blocked influenza replication at a concentration of 1 ng/ml with a 3-fold reduction in NS1 protein expression (Fig. 4C; lane 7) and a 7-fold reduction in virus titer by plaque assay (Fig. 4D).

In another experiment, A549 cells were treated with a single dose of 5'ppp-SEQ ID NO: 1 pre- (-24 hours, -8 hours, -4 hours) and post- (+1 hour, +4 hours) influenza challenge. As shown by NS1 expression, pre-treatment with 10 ng/ml 5'ppp-SEQ ID NO: 1 for 8 hours caused a 100-fold reduction in influenza NS1 expression (Fig. 4E, lane 9). Pre-treatment for 4 hours was also effective and resulted in an 8-fold reduction in NS1 (Fig. 4E; lane 10). Additionally, treatment at both 1 and 4 hours post-infection also reduced influenza NS1 expression by 2-fold (Fig. 4E; lanes 11 and 12).

In another experiment siRNA was used to silence RIG-I or IFN α / β receptor in A549 cells that were later infected with influenza. Note that ISG's were not induced by the siRNA (Fig. 4F, lanes 3 vs. 6). 5'ppp-SEQ ID NO: 1 treatment did not inhibit NS1 expression in these infected cells (Fig. 4F; lanes 5 vs. 6). In cells with IFN α / β R expression silenced, there was no IFIT1 or RIG-I expression following treatment with IFN α -2b (Fig. 4G; lane 6). Expression of ISGs was only partially reduced following treatment with 5'ppp-SEQ ID NO: 1. There was a 2.2- fold reduction of IFIT1 in cells with a silenced with IFN α / β R siRNA relative to the negative control siRNA (Fig. 4G; lane 5 vs. 2). However, in those cells, 5'ppp-SEQ ID NO: 1 treatment reduced viral NS1 expression by 2.4-fold (Fig. 4F; lane 9 vs. 8).

Example 5 – 5'-ppp-SEQ ID NO: 1 activates innate immunity and protects mice from lethal influenza infection.

C57Bl/6 mice were inoculated intravenously with 5'ppp-SEQ ID NO: 1 in complex with *in vivo*-jetPEI™ transfection reagent. 5'ppp-SEQ ID NO: 1 stimulated a potent immune response in vivo characterized by IFN α and IFN β secretion in the serum and lungs (Fig. 9A) as well as antiviral gene up-regulation (Fig. 9B). Following intravenous injection, serum IFN β levels were increased ~20-fold compared to basal levels, as early

as 6 hours post administration (Figure 9A top left panel). The immune activation observed in vivo correlated with an early and transient recruitment of neutrophils to the lungs along with a more sustained increase in macrophages and dendritic cells (Figure 9C).

In another experiment, mice were treated with 25 µg of 5'ppp-SEQ ID NO: 1 as described above 24 hours before (day -1), and on the day of infection (day 0) with a lethal inoculum of H1N1 A/PR/8/34 Influenza. All untreated, infected mice succumbed to infection by day 11, but all 5'ppp-SEQ ID NO: 1 -treated mice fully recovered (Fig. 5A). Overall weight loss was similar between the two groups (Fig. 5B), although a delay of 2-3 days of the onset of weight-loss was observed in 5'ppp-SEQ ID NO: 1 -treated animals. Treated mice fully recovered within 12-14 days (Fig. 5B). Influenza replication in the lungs was monitored by a plaque assay performed throughout the course of infection. Virus titers in the lungs of untreated mice peaked at day 3 post-infection (Fig. 5C) with a decrease in virus titer observed at day 9 post-infection. In the 5'-ppp-SEQ ID NO: 1 treated animals, influenza virus replication in the lungs was inhibited within the first 24-48 hours (Fig. 5C; Day 1). By day 3, virus titers in the lung had increased, although influenza titers were still ~10-fold lower compared to titers in untreated mice (Fig. 5C; Day 3). By day 9, the 5'ppp-SEQ ID NO: 1 had a sufficiently low viral titer to indicate that they controlled the infection. Continuous administration of 5'ppp-SEQ ID NO: 1 at 24 hour intervals post-infection had an additive therapeutic effect that further delayed viral replication (Fig. 5D; 3 versus 2 doses of 5'ppp-SEQ ID NO: 1). Administration of 5'ppp-SEQ ID NO: 1 therapeutically also controlled influenza viral replication. Administration of 5'ppp-SEQ ID NO: 1 at day 1 and day 2 following infection reduced viral lung titers by ~10-fold (Fig. 5E).

IFNβ release did not occur in MAVS^{-/-} mice treated with 5'ppp-SEQ ID NO: 1 but did occur in TLR3^{-/-} mice treated with 5'ppp-SEQ ID NO: 1 indicating that IFNβ release by 5'ppp-SEQ ID NO: 1 is dependent on an intact RIG-I pathway (Fig. 5F). MAVS^{-/-} mice

treated with 5'ppp-SEQ ID NO: 1 did not control influenza lung titers (5-fold increase vs. wt mice) and the titer was comparable to untreated wt mice (Fig. 5G).

In another experiment, IFN α / β R^{-/-} mice were treated with 5'ppp-SEQ ID NO: 1 and infected with influenza H1N1 virus and compared to untreated infected IFN α / β R^{-/-}. While untreated IFN α / β R^{-/-} animals succumbed to infection, 40% of the animals that received 5'ppp-SEQ ID NO: 1 treatment survived, suggesting that an IFN-independent effect of 5'ppp-SEQ ID NO: 1 provided some protection.

Example 6 - 5'ppp-SEQ ID NO: 1 treatment limits influenza-mediated pneumonia

To further evaluate the effect of 5'ppp-SEQ ID NO: 1 administration on influenza-mediated pathology, histological sections of lungs from mice treated with 5'ppp-SEQ ID NO: 1 were compared to untreated mice. 5'ppp-SEQ ID NO: 1 treatment alone (no infection) was characterized by a modest and rare leukocyte-to-endothelium attachment. Mixed leukocyte populations (mononuclear/polymorphonuclear) infiltrated the perivascular space at 24h after injection but the infiltration resolved and was limited to endothelial cell attachment at 3 and 8 days after intravenous administration (Fig. 6A). Influenza virus infection without treatment with 5'ppp-SEQ ID NO: 1 induced severe and extensive inflammation and oedema in the perivascular space and the bronchial lumen at day 3 post-infection.

In animals infected with Influenza virus and treated with 5'ppp-SEQ ID NO: 1, influenza infection triggered a mild and infrequent inflammation that did not extend to the bronchial lumen at day 3 post-infection. Epithelial degeneration and loss of tissue integrity were more severe in the lungs of untreated, infected animals and correlated with epithelial hyperplasia observed at later times, when compared to the lungs of animals treated with 5'ppp-SEQ ID NO: 1. Inflammation and epithelial damage progressed in untreated mice by day 8 (Fig. 6B), and correlated with the increased viral titer in the lungs described above. Inflammation and epithelial damage was consistently less apparent in influenza infected mice treated with 5'ppp-SEQ ID NO: 1. The surface area of the lungs affected by pneumonia was significantly reduced in 5'ppp-SEQ ID NO: 1

-treated mice compared to infected, but untreated mice. On day 3, 16% of the surface area of infected 5'ppp-SEQ ID NO: 1 treated mice was affected by pneumonia while 35% of the surface area of infected untreated mice. By day 8, 41% of the surface area of 5'ppp-SEQ ID NO: 1 treated mice was affected by pneumonia vs 73% of the surface area of infected untreated mice (Fig. 6C; bottom panel). Overall, influenza-mediated pneumonia was less severe in animals administered 5'ppp-SEQ ID NO: 1 before infection with influenza.

Example 7 – Materials and Methods

Materials and Methods in this Example are in reference to Examples 1-6 above.

In vitro synthesis of 5'ppp-SEQ ID NO: 1: In vitro transcribed RNA was prepared using the Ambion MEGAscript® T7 High Yield Transcription Kit according to the manufacturer's instruction. The template included two complementary viral sequences operably linked to a T7 promoter that were annealed at 95 °C for 5 minutes and cooled down gradually over night. The in vitro transcription reactions proceeded for 16 hours. 5'ppp-SEQ ID NO: 1 was purified and isolated using the Qiagen miRNA Mini® Kit. An oligoribonucleotide equivalent to SEQ ID NO: 1 lacking a 5'ppp moiety was purchased from Integrated DNA Technologies, Inc. A secondary structure of 5'ppp-SEQ ID NO: 1 was predicted using the RNAfold WebServer (University of Vienna, Vienna, Austria).

Cell culture, transfections, and luciferase assays: A549 cells were grown in F12K media supplemented with 10% FBS and antibiotics. To generate a stable MAVS-negative cell line, a MAVS specific shRNA was used (Xu LG *et al*, 2005 *supra*). Plasmids pSuper VISA® RNAi and pSuper® control shRNA were transfected in A549 cells using Lipofectamine 2000® according to the manufacturer's instructions. MAVS-negative cells were selected beginning at 48 hours for approximately 2 weeks in F12K containing 10% FBS, antibiotics, and 2µg/ml puromycin. Mouse endothelial fibroblasts (MEF's) were grown in DMEM supplemented with 10% FBS, non-essential amino acids, and L-Glutamine. RIG-I^{-/-} MEFS are described in Kato H *et al*, Immunity **23**, 19-28 (2005); (incorporated by reference herein). MDA5^{-/-}, TLR3^{-/-}, and TLR7^{-/-} MEFS are described in

Gitlin L *et al*, *Proc Natl Acad Sci USA* **103**, 8459-3464 (2006) and McCartney S *et al*, *J Exp Med* **206**, 2967-2976 (2009), both of which are incorporated by reference herein.

Lipofectamine RNAiMax[®] was used for transfections in A549 according to manufacturer's instructions. For luciferase assays, transfections were performed in wt and RIG-I^{-/-}; wild type, MDA5^{-/-}, TLR3^{-/-}, and TLR7^{-/-} MEFs using Lipofectamine 2000[®] and jetPRIME[®]. Plasmids encoding GFP-RIG-I, IRF-7, pRLTK, IFN α 4/pGL3 and IFN β /pGL3 were previously described in Zhao T *et al*, *Nat Immunol* **8**, 592-600 (2007). The IFN λ 1-luciferase reporter is described in Osterlund PI *et al*, *J Immunol* **179**, 3434-3442 (2007) which is incorporated by reference herein.

MEFs were co-transfected with 200ng pRLTK reporter (*Renilla* luciferase for internal control), 200 ng of reporter gene constructs: IFN α 4, IFN β , and IFN λ 1, together with 5'ppp-SEQ ID NO: 1 (500ng/ml) or 100ng of a plasmid encoding a constitutively active form of RIG-I (Δ RIG-I) (Yoneama M *et al* *Nat Immunol* **5**, 730-737 (2004), incorporated by reference herein.) IRF7 plasmid (100 ng) was added for transactivation of the IFN α 4 promoter. At 24h after transfection, reporter gene activity was measured by a Promega Dual-Luciferase Reporter Assay according to manufacturer's instructions. Relative luciferase activity was measured as fold induction relative to the basal level of the reporter gene.

Immunoblot analyses: Whole cell extracts (40 μ g) were separated in 8% acrylamide gel by SDS-PAGE and were transferred to a nitrocellulose membrane at 4 °C for 1 hour at 100 volts in a buffer containing 30mM Tris, 200mM glycine and 20% methanol. Membranes were blocked for 1h at room temperature in 5% dried milk (wt/vol) in PBS and 0.1% Tween-20 (vol/vol) and probed with primary antibodies to IRF3 phosphorylated at Ser396, IRF3, RIG-I, ISG56, STAT1 phosphorylated at Tyr701, STAT1, NS1, I κ B α phosphorylated at Ser32, I κ B α , NOXA, cleaved Caspase 3, PARP, and β -actin. Antibody signals were detected by chemiluminescence using secondary antibodies conjugated to horseradish peroxidase and an Amersham Biosciences ECL detection kit.

IRF3 dimerization: Whole cell extracts were prepared in NP-40 lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 30mM NaF, 5mM EDTA, 10% glycerol, 1.0mM Na₃VO₄, 40mM β -glycerophosphate, 0.1mM phenylmethylsulfonyl fluoride, 5 μ g/ml of each leupeptin, pepstatin, and aproptinin, and 1% Nonidet P-40). Whole cell extracts were then electrophoresed on 7.5% native acrylamide gel, which was pre-run for 30 min at 4° C. The upper chamber buffer was 25mM Tris at pH 8.4, 192mM glycine, and 1% sodium deoxycholate and the lower chamber buffer (25mM Tris at pH 8.4 and 192mM glycine). Gels were soaked in SDS running buffer (25mM Tris, at pH 8.4, 192mM glycine, and 0.1% SDS) for 30 min at 25 °C and were then transferred to nitrocellulose membrane. Membranes were blocked in PBS containing 5% milk (wt/vol) and 0.05% Tween®-20 (vol/vol) for 1 hour at 25 °C and blotted with an antibody against IRF3. Antibody signals were detected by chemiluminescence using secondary antibodies conjugated to horseradish peroxidase and an Amersham Biosciences ECL detection kit.

ELISA: The release of human IFN α (multiple subunits) and IFN β in culture supernatants of A549, and murine IFN β in mouse serum were measured using the appropriate ELISA kits from PBL Biomedical Laboratories according to manufacturer's instructions.

Primary cell isolation: PBMCs were isolated from freshly collected human blood using a Cellgro® Lymphocyte Separation Medium according to manufacturer's instructions. After isolation, total PBMCs were frozen in heat-inactivated FBS with 10% DMSO. On experimental days, PBMCs were thawed, washed and placed at 37 °C for 1 hour in RPMI with 10% FBS supplemented with Benzonaze® nuclease to prevent cell clumping.

Virus production and infection VSV-GFP, which harbors the methionine 51 deletion in the matrix protein-coding sequence (Stojdl D *et al*, *Cancer Cell* **4**, 263-275 (2003) was grown in Vero cells, concentrated from cell-free supernatants by centrifugation, and titrated by a standard plaque assay as described previously in Tumilasci VF *et al*, *J Virol* **82**, 8487-8499 (2008), incorporated by reference herein. The

recombinant vaccinia-GFP virus VVE3L-REV), a revertant strain of the E3L deletion mutant is described in Myskiw C *et al*, *J Virol* **85**, 12280-12291 (2011) and Arseniob J *et al*, *Virology* **377**, 124-132 (2008).

Dengue virus serotype 2 (DENV-2) strain New Guinea C was grown in C6/36 insect cells for 7 days. Cells were infected at a MOI of 0.5, and 7 days after infection, cell supernatants were collected, clarified and stored at -80 °C. Titers of DENV stocks were determined by serial dilution on Vero cells and intracellular immunofluorescent staining of DENV E protein at 24 hours post-infection. Titer is given as infectious units per ml. In infection experiments, both PBMCs and A549 cells were infected in a culture media without FBS for 1 hour at 37 °C and then incubated with complete medium for 24 hours prior to analysis.

HIV-GFP virus is an NL4-3 based virus designed to co-express Nef and eGFP from a single bicistronic RNA. HIV-GFP particles were produced by transient transfection of pBR43leG-nef+ plasmid into 293T cells as described in Schindler M *et al*, *J Virol* **79**, 5489-5498 (2005) and Schindler M *et al*, *J Virol* **77**, 10548-10556 (2003), both of which are incorporated by reference herein. 293T cells were transfected with 22.5 µg of pBR43leG-nef+ plasmid by polyethylenimine precipitation. Media was replaced 14 to 16 hours post-transfection, viral supernatants were harvested 48 hours later, cleared by low-speed centrifugation and filtered through a 0.45 µm low binding protein filter. High-titer viral stocks were prepared by concentrating viral supernatants 100-fold through filtration columns. These were then stored at -80°C. Viral titers were determined by p24 level (ELISA) and TCID₅₀. A set of 10-fold serial dilutions of concentrated viral supernatants were used to infect PBMCs pre-activated for 3 days with 10 µg/ml of PHA. Four days after infection half the media was replaced. Seven days after infection, supernatants were harvested and titered by ELISA. TCID₅₀T was calculated by the Reed–Muench method.

CD14⁺ monocytes were negatively selected using the EasySep® Human Monocytes Enrichment Kit as per manufacturer's instructions. Isolated cells were

transfected with 5'ppp-SEQ ID NO: 1 (100 ng/ml) using Lyovect (Invitrogen) according to the manufacturer's protocol. Supernatants were harvested 24 hours after stimulation and briefly centrifuged to remove cell debris. CD4⁺ T cells were isolated using EasySep[®] Human CD4⁺ T cells Enrichment Kit according to the manufacturer's instructions. Purified CD14⁺ monocytes and CD4⁺ T cells were allowed to recover for 1 hour in RPMI containing 10% FBS at 37 °C with 5% CO₂ before experiments. For HIV infection, anti-CD3 antibodies at 0.5 µg/ml were immobilized for 2 hours in a 24-well plate. CD4⁺ T cells were then added along with an anti-CD28 antibody (1 µg/ml) to allow activation of T cells for 2 days. After activation, cells were incubated for 4 hours with supernatant of monocytes stimulated with 5'ppp-SEQ ID NO: 1 and infected with HIV-GFP at an MOI of 0.1. Supernatant from the monocytes was left for another 4h before adding complete medium.

HCV RNA was synthesized using the Ambion MEGAscript[®] T7 High Yield Transcription Kit using linearized pJFH1 DNA as a template. Huh7 cells were electroporated with 10 mg of HCV RNA. At 5 days post-transfection, supernatants containing HCV (HCVcc) were collected, filtered (0.45 µm) and stored at -80 °C. Huh7 or Huh7.5 cells were pre-treated with 5'-ppp-SEQ ID NO: 1 (10 ng/ml) for 24h. Cell culture supernatants containing soluble factors induced following 5'-ppp-SEQ ID NO: 1 treatment were removed and kept aside during infection. Cells were washed once with PBS and infected with 0.5 ml of undiluted HCVcc for 4 hours at 37 °C. After infection, supernatant from 5'ppp-SEQ ID NO: 1 treated cells was added back. At 48 hours post infection, whole cell extracts were prepared and the expression of HCV NS3 protein was detected by Western blot.

Influenza H1N1 strain A/Puerto Rico/8/34 was amplified in Madin-Darby canine kidney (MDCK) cells and virus titer determined by standard plaque assay (Szretter KJ *et al*, *Curr Protoc Microbiol* Chapter 15.1 (2006), incorporated by reference herein.) Cells were infected in 1 ml medium without FBS for 1 hour at 37 °C. Inoculum was aspirated and cells were incubated with complete medium for 24 hours, unless otherwise

indicated, prior to analysis. For viral infections, supernatants containing soluble factors induced by treatment with 5'ppp-SEQ ID NO: 1 were removed and kept aside during infection. Cells were washed once with PBS and infected in a small volume of medium without FBS for 1h at 37° C; then supernatant was then added back for the indicated period of time.

Flow cytometry: The percentage of cells infected with VSV, Vaccinia and HIV was determined based on GFP expression. The percentage of cells infected with Dengue was determined by standard intracellular staining. Cells were stained with a mouse IgG2a monoclonal antibody specific for DENV-E-protein (clone 4G2) followed by staining with a secondary anti-mouse antibody coupled to PE. PBMCs infected with DENV2 were first stained with anti-human CD14 AlexaFluor® 700 Ab. Cells were analyzed on a LSRII® flow cytometer. Compensation calculations and cell population analysis were done using FACS® Diva.

In vivo administration of 5'ppp-SEQ ID NO: 1 and influenza infection model: C57Bl/6 mice (8 weeks) were obtained from Charles River Laboratories. MAVS^{-/-} mice on a mixed 129/SvEv-C57Bl/6 background were obtained from Z. Chen (The Howard Hughes Medical Institute, US). TLR3^{-/-} mice were obtained from Taconic. For intracellular delivery, 25ug of 5'ppp-SEQ ID NO: 1 was complexed with In vivo-JetPEI® at an N/P ratio of 8 as per manufacturer's instructions and administered intravenously via tail vein injection. Unless otherwise indicated, 5'ppp-SEQ ID NO: 1 was administered on the day prior to infection (Day -1) and also on the day of infection (Day 0). Mice infected intra-nasally with 500 pfu of Influenza A/PR/8/34 under 4% isoflurane anesthesia. For viral titers, lungs were homogenized in DMEM (20% wt/vol) and titers were determined by standard plaque assay as previously described in Szretter KJ *et al*, 2006 *supra*. Confluent Madin-Darby Canine Kidney Cells (MDCK) were incubated with 250 µL of serial 10-fold dilutions of homogenized lung sample for 30 minutes. The sample was aspirated, and cells overlaid with 3 ml of 1.6% agarose in DMEM. Plaques were fixed and counted 48 hours later.

Histology and pathology: All five lobes of the lungs were collected and fixed in neutral-buffered formalin for 24 hours. The tissues were paraffin-embedded and 4 μ m sections were prepared using a microtome. Hematoxylin and eosin staining (H&E) were performed using standard protocols and analyzed by an independent veterinary pathologist.

Example 8 - 5'ppp-SEQ ID NO: 1 inhibits DENV infection

5'ppp-SEQ ID NO:1 inhibits DENV infection. To determine the capacity of the 5'ppp-SEQ ID NO:1 RIG-I agonist to induce a protective antiviral response to DENV infection, A549 cells were challenged with DENV at different multiplicities of infection (MOI); infection. Replication was monitored by flow cytometry, RT-qPCR, plaque assay, and immunoblotting (Figure 10A to 10F). DENV established infection in A549 cells. The infection was completely abrogated in cells pretreated with 1 ng/ml of 5'ppp-SEQ ID NO: 1 (Figure 10A). A similar antiviral effect was observed at higher concentrations of 5'ppp-SEQ ID NO: 1 (10 ng/ml). The antiviral effect was dependent on the 5'ppp moiety because transfection of cells with the identical RNA sequence lacking the 5'ppp did not prevent DENV infection (Figure 10B). Pretreatment of cells with 5'ppp-SEQ ID NO: 1 also led to an 8.5-fold decrease in DENV RNA synthesis (Figure 10C). Release of infectious DENV was completely suppressed by 5'ppp-SEQ ID NO: 1 treatment (4.3×10^6 PFU/ml in untreated cells versus undetectable in 5'ppp-SEQ ID NO: 1 treated cells) (Figure 10D). This led to a complete inhibition of DENV E protein expression (Figure 10D, lane 3). To compare the effect of 5'ppp-SEQ ID NO: 1 to that of the dsRNA ligand poly(I:C), A549 cells were pretreated with 5'ppp-SEQ ID NO: 1 or poly(I:C) (0.1 to 1 ng/ml) and subsequently challenged with DENV (Figure 10E). Treatment with 1 ng/ml of 5'ppp-SEQ ID NO: 1 almost completely suppressed DENV infection. At the same concentration, only a 1.8-fold decrease of the number of DENV-infected cells was observed with poly(I:C) treatment (Figure 10E). Cytosolic delivery of dsRNA by transfection was required in A549 cells, as demonstrated by the absence of a protective antiviral effect in cells in medium to which 5 μ g/ml of 5'ppp-SEQ ID NO: 1 or poly(I:C) had just been added (Figure 10E).

To determine whether pretreatment with 5'ppp-SEQ ID NO: 1 maintained a protective effect, A549 cells were transfected with 5'ppp-SEQ ID NO: 1 prior to DENV challenge and the virus was allowed to replicate up to 72 h post infection (Figure 10F). The combination treatment completely inhibited DENV infection at all time points for up to 72 h post infection (Figure 10F). The viability of uninfected cells and cells protected from infection by 5'ppp-SEQ ID NO: 1 was indistinguishable (Figure 10G). Altogether, these results demonstrate the antiviral potential of 5'ppp-SEQ ID NO: 1 against DENV infection in nonimmune cells.

To assess the potential of 5'ppp-SEQ ID NO: 1 as a postinfection treatment, A549 cells were first infected with DENV, subsequently treated with 5'ppp-SEQ ID NO: 1 at 4 h and 8 h after infection, and analyzed 48 h later to detect DENV infection. Infection was almost completely inhibited even when cells were treated at 8 hours post infection, as shown by the 12.4-fold reduction of the number of DENV-infected cells (Figure 11A). This suggests that as DENV replicates over time 5'ppp-SEQ ID NO: 1 prevents further spread of the virus by protecting uninfected cells and clearing virus from infected cells. The observed effects of 5'ppp-SEQ ID NO: 1 on DENV infection were confirmed by RT-qPCR, yielding a 3.6-fold (+ 4 hours) and 10.8-fold (+8 hour) decrease in DENV viral RNA levels at 48 h post infection. (Figure 11B). Cell viability was not significantly affected by a 24-h 5'ppp-SEQ ID NO: 1 treatment and an approximate 20% decrease in viability was observed at 48 h p.i. in cells protected from infection by 5'ppp-SEQ ID NO: 1 (Figures 11C and 11D).

To investigate the antiviral response triggered by 5'ppp-SEQ ID NO: 1, various signaling parameters were monitored by immunoblotting and RT-qPCR in cells treated with increasing doses of 5'ppp-SEQ ID NO: 1 in the presence or absence of DENV infection (Figure 11E and 11F). Interferon signaling was detected by immunoblotting in 5'ppp-SEQ ID NO: 1 treated cells, both in the presence or absence of DENV, as demonstrated by increased STAT1 Tyr701 phosphorylation and ISG expression of STAT1, RIG-I, and IFIT1 (Figure 11E, lanes 2 to 8). Although DENV can evade the host innate

response, a significant inhibition of IFN signaling was not observed based on the expression of antiviral markers STAT1, RIG-I, and IFIT1 in infected or uninfected cells (Figure 11E, lanes 2 to 8).

5'ppp-SEQ ID NO: 1 treatment elicited a strong antiviral response in uninfected and DENV-infected A549 cells (Figure 11E), and delivery of 5'ppp-SEQ ID NO: 1 at 4 hours post infection potently stimulated type I IFN and inflammatory responses via the upregulation of genes, such as those of IFN- α , IFN- β , IL-6, and IL-1 α (Figure 11F).

Example 9 - 5'ppp-SEQ ID NO: 1 restricted DENV infection requires an intact RIG-I pathway

Introduction of RIG-I siRNA (10 and 30 pmol) into A549 cells severely reduced RIG-I as well as IFIT1 induction in response to 5'ppp-SEQ ID NO: 1 treatment (Figure 12A, lanes 5 to 8). Induction of the type I and type III IFNs, as well as the inflammatory response, were all dependent on intact RIG-I signaling, since the mRNA levels of IFN- α , IFN- β , IL-29, and tumor necrosis factor alpha (TNF- α) were drastically decreased in the absence of RIG-I expression (Figure 12B). To explore the respective involvement of RIG-I, TLR3, and MDA5 in the 5'ppp-SEQ ID NO: 1 mediated anti-DENV effect, the expression of these immune sensors was knocked down in A549 cells by siRNA (Figure 12C). While impairing RIG-I expression completely suppressed the 5'ppp-SEQ ID NO: 1-mediated antiviral effect, this was not the case upon knockdown of TLR3/MDA5 (Figure 12C). The efficacy of poly(I:C) in preventing DENV infection was reduced to a larger extent in the absence of TLR3/MDA5 than in the absence of RIG-I, suggesting a predominant role for TLR3/MDA5 in mediating poly(I:C) antiviral effect in A549 cells (Figure 12C). To demonstrate that the antiviral activity of 5'ppp-SEQ ID NO: 1 against DENV relies on a functional RIG-I axis, the expression of RIG-I, STING, MAVS, and TBK1 was depleted in A549 cells using specific siRNAs. In addition, suitable knockout MEFs were used (Figure 12D, 12E, and 12F). Following 5'ppp-SEQ ID NO: 1 treatment, DENV viral replication was assessed by flow cytometry. Whereas about 35% of A549 cells were infected with DENV in the untreated population, the absence of RIG-I led to a 1.5-fold increase in the

number of infected cells (Figure 12D). Transient knockdown of RIG-I resulted in the abrogation of the protective response induced by 5'ppp-SEQ ID NO: 1 in control cells (Figure 12D), whereas the absence of STING did not affect DENV infection and did not significantly reduce the 5'ppp-SEQ ID NO: 1 -induced antiviral response (Figure 12D). Similar results were observed with A549 cells depleted for the mitochondrial adaptor MAVS. Depletion of MAVS strongly reduced the 5'ppp-SEQ ID NO: 1 -mediated protective antiviral response (Figure 12E). Finally, TBK1-deficient MEFs were more susceptible to DENV infection than wild-type MEFs and were not responsive to 5'ppp-SEQ ID NO: 1 treatment, as demonstrated by the high level of DENV infection (Figure 12F). In conclusion, 5'ppp-SEQ ID NO: 1 treatment efficiently generates a RIG-I/MAVS/TBK1-dependent antiviral response that limits DENV infection in vitro.

Example 10 - 5'ppp-SEQ ID NO: 1 generates an IRF3-dependent and IFNAR/STAT1-independent antiviral protective effect

To determine whether the potent RIG-I activation brought about by 5'ppp-SEQ ID NO: 1 could compensate for the type I and type III IFN response, expression of the type I IFN receptor (IFN- α/β R) as well as the type III IFN receptor (IL-28R plus IL-10R β) was knocked down using siRNA in A549 cells (Figures 13A, 13B and 13C). Expression of both type I and III IFN receptor was efficiently reduced, as shown by the downregulation of IFNAR1 (IFN α/β R α chain), IFNAR2 (IFN- α/β R β chain), and IL-28R mRNA expression levels (Figure 13A). Furthermore, knockdown of type I IFN signaling was highly efficient, as demonstrated by the reduction of IFIT1 and RIG-I induction following IFN- α 2b stimulation (6.2-fold reduction of IFIT1 versus control siRNA [siCTRL]; Figure 13B, lane 3 versus lane 6). Knocking down the type III IFN receptor did not interfere with the ability of 5'ppp-SEQ ID NO: 1 and IFN- α 2b to induce IFIT1 and RIG-I expression (Figure 13B, lanes 2 and 3 versus lanes 8 and 9).

Induction of IFIT1 but not RIG-I was only partially reduced following 5'ppp-SEQ ID NO: 1 treatment in the absence of type I IFN receptor (1.6-fold reduction of IFIT1 versus siCTRL; Figure 13B, lane 2 versus lane 5), suggesting that certain ISGs were upregulated

by 5'ppp-SEQ ID NO: 1 in an IFN-independent manner. Knocking down expression of both type I and type III IFN receptors did not limit IFIT1 induction by 5'ppp-SEQ ID NO: 1, as the increase of IFIT1 was only reduced 1.9 times compared to the siRNA control (Figure 13B). This type I and III IFN-independent activation of the innate system was sufficient to suppress DENV infection in A549 cells stimulated with a higher (10 ng/ml) but not a low dose (0.1 to 1 ng/ml) of 5'ppp-SEQ ID NO: 1 (Figure 13C). To further confirm that type I IFN signaling was not necessarily required to mediate an immune response to 5'ppp-SEQ ID NO: 1, STAT1 was depleted in A549 cells using siRNA (Figure 13D, lanes 5 to 8). The increased expression of IFIT1 following 5'ppp-SEQ ID NO: 1 treatment was not impacted by the absence of the STAT1 transcription factor (Figure 13D, lanes 2 to 4 versus lanes 6 to 8). The STAT1-independent induction of the antiviral response was sufficient to block DENV infection in A549 cells stimulated with a high 5'ppp-SEQ ID NO: 1 concentration (Figure 13E). Finally, to determine which IRF transcription factor downstream of RIG-I was involved in the antiviral protective effect, IRF1, IRF3, and IRF7 expression was knocked down using siRNA (Figure 13F). Depletion of these different transcription factors was highly efficient, as shown in Figure 13F. Only IRF3 knockdown resulted in inhibition of the protective antiviral response generated by 5'ppp-SEQ ID NO: 1 treatment. Indeed, the absence of either IRF1 or IRF7 did not impair 5'ppp-SEQ ID NO: 1-mediated antiviral protection (Figure 13G). Altogether, these data demonstrate that the 5'ppp-SEQ ID NO: 1-mediated anti-DENV effect in vitro is largely independent of the type I or type III IFN responses but requires the activation of a functional RIG-I/IRF3 axis to mediate its protective effect.

Example 11 – A protective antiviral response against DENV in primary human myeloid cells

Cells of the myeloid lineage, including monocyte/macrophages and dendritic cells, are the primary target cells for DENV infection among human peripheral blood mononuclear immune cells. Severe and potentially lethal manifestations associated with secondary DENV infection are often related to antibody-dependent enhancement (ADE)

of infection. To address the impact of 5'ppp-SEQ ID NO: 1 on ADE-mediated DENV infection, we demonstrated, using isolated human monocytes, that anti-DENV E 4G2 antibody increased DENV infectivity from 16.4% to 24.4% (Figure 14A), whereas a control isotype IgG2a antibody did not significantly increase viral infectivity (Figure 14A). Both primary and ADE DENV infections were completely suppressed by 5'ppp-SEQ ID NO: 1 treatment (16.4% and 24.4% in untreated cells versus 0.1% and 0.3% in 5'ppp-SEQ ID NO: 1-treated cells, respectively).

Similarly, in primary human MDDC, which are highly permissive to DENV, infection decreased 8.4-fold in the presence of 5'ppp-SEQ ID NO: 1 in combination with Lyovec (Figure 14B), and cell viability was not affected by increasing concentrations of 5'ppp-SEQ ID NO: 1 (Figure 14C). MDDC treated with 5'ppp-SEQ ID NO: 1 at 4 hours post infection, were assessed for markers of activation of the innate immune response (Figure 14D). Increased levels of phosphorylated IRF3 and STAT1 were observed, and a 2- to 10-fold increase in the expression of ISG RIG-I and IFIT-1 following 5'ppp-SEQ ID NO: 1 treatment were observed (Figure 14D, lane 2). A similar response was observed with DENV infection alone (Figure 14D, lane 3). The innate DNA sensor STING was known to be cleaved and inactivated by DENV NS2/3 protease. In the experiments disclosed herein, STING expression was not modulated by 5'ppp-SEQ ID NO: 1 or DENV infection alone (Figure 14D, lane 2 and 3). Also, postinfection treatment with 5'ppp-SEQ ID NO: 1 moderately increased the levels of the following markers of the innate immune response compared to virus alone: phospho-STAT1 (3-fold increase), STAT1 (1.4-fold increase), IFIT1 (1.3-fold increase), and RIG-I (1.3-fold increase) (Figure 14D, lanes 3 and 4). Surprisingly, 5'ppp-SEQ ID NO: 1 did not further increase the level of phospho-IRF3 compared to DENV infection alone (Figure 14D, lane 3 and 4), an observation that is in part attributable to the early and transient kinetics of IRF3 phosphorylation. These data demonstrate that RIG-I activation by 5'ppp-SEQ ID NO: 1 triggers an immune response capable of inhibiting DENV in both primary and ADE models of infection.

Example 12 - 5'ppp-SEQ ID NO: 1 treatment inhibits CHIKV replication in a RIG-I-dependent manner.

To explore the potential of 5'ppp-SEQ ID NO: 1 to prevent CHIKV infection, human fibroblast MRC-5 cells were pretreated with increasing concentrations of 5'ppp-SEQ ID NO: 1 prior to challenge with a CHIKV LS3-GFP reporter virus (Figure 15A). CHIKV replication was strongly inhibited in a dose-dependent manner in cells treated with 5'ppp-SEQ ID NO: 1 one hour prior to infection (Figure 15A); as little as 1 ng/ml completely blocked CHIKV EGFP reporter gene expression, and the 5'ppp-SEQ ID NO: 1 concentration required to completely block CHIKV replication in MRC-5 cells was 10-fold lower than that required to inhibit DENV in A549 cells. It is currently unclear whether this is due to virus-specific immune evasion or cell type-specific differences, as CHIKV does not replicate in A549 cells. Also, introduction of control RNA lacking the 5'-triphosphate moiety only led to a minor reduction of GFP reporter gene expression in CHIKV LS3-GFP-infected cells (Figure 15A). Cell viability, monitored in parallel, was not significantly affected by transfection of either 5'ppp-SEQ ID NO: 1 or control RNA lacking the 5' triphosphate (Figure 15B). Analysis of intracellular RNA of CHIKV-infected cells pretreated 5'ppp-SEQ ID NO: 1 or control RNA showed that treatment with 0.1 ng/ml 5'ppp-SEQ ID NO: 1 reduced CHIKV positive- and negative-strand RNA accumulation to minimally detectable levels (Figure 15C), and at higher doses of 5'ppp-SEQ ID NO: 1 was undetectable. Transfection of cells with control RNA prior to infection had no significant effect on the accumulation of CHIKV RNA (Figure 15C). To determine the effect 5'ppp-SEQ ID NO: 1 treatment on the expression of CHIKV nonstructural proteins (translated from genomic RNA) and structural proteins (translated from the sgRNA), cells were pretreated with 5'ppp-SEQ ID NO: 1 or control RNA and infected with CHIKV, and nsP1 and E2 expression was analyzed by Western blotting (Figure 15D). Transfection of 0.1 ng/ml 5'ppp-SEQ ID NO: 1 led to a 4-fold reduction in nsP1 expression and an 8-fold reduction in E2 expression. Higher doses of 5'ppp-SEQ ID NO: 1 reduced nsP1 and E2 expression over 30-fold (Figure 15D). Transfection of control RNA lacking the 5'

triphosphate had no noticeable effect on CHIKV protein expression (Figure 15D). Finally, the effect of 5'ppp-SEQ ID NO: 1 treatment on the production of infectious progeny was determined. Compared to untreated cells, transfection of MRC-5 cells with 0.1 ng/ml of 5'ppp-SEQ ID NO: 1 one hour prior to CHIKV infection led to a 1 log reduction in virus titer, while transfection with 1 ng/ml and 10 ng/ml 5'ppp-SEQ ID NO: 1 reduced viral progeny titers by 2 and 3 logs, respectively (Figure 15E). Transfection of control RNA lacking the 5' triphosphate did not significantly affect CHIKV progeny titers (Figure 15E).

To determine which innate immune pathways are involved in the 5'ppp-SEQ ID NO: 1 mediated inhibition of CHIKV replication, several key proteins of the IFN signaling pathway (RIG-I, STAT1, and STING) were depleted in MRC-5 cells using siRNAs. Knockdown levels were assessed by Western blotting (Figure 15G). Subsequently, cells depleted for RIG-I, STAT1, or STING were treated with 5'ppp-SEQ ID NO: 1 and infected 1 h later with CHIKV LS3-GFP (Figure 15F). CHIKV-driven GFP reporter gene activity was reduced to almost background levels in 5'ppp-SEQ ID NO: 1 -treated cells that were depleted for STAT1 and STING, suggesting these proteins are not involved in the 5'ppp-SEQ ID NO: 1 -mediated antiviral response to CHIKV. In contrast, CHIKV replication was observed in cells depleted of RIG-I and treated with 5'ppp-SEQ ID NO: 1, although EGFP reporter gene expression was 30% of that in untreated cells transfected with scrambled (or RIG-I-targeting) siRNAs (Figure 15F). This partial recovery of replication might be due to incomplete knockdown of RIG-I in a fraction of the cells and/or paracrine IFN signaling of those cells, which could affect CHIKV replication of RIG-I-depleted cells. CHIKV replication in cells depleted for RIG-I, STAT1, or STING, but not treated with 5'ppp-SEQ ID NO: 1, was similar or slightly increased compared to that of cells transfected with a scrambled control siRNA. In parallel, the siRNA-treated cells were transfected with 1 ng/ml 5'ppp-SEQ ID NO: 1, and 24 h later the IFN signaling response was analyzed by monitoring the upregulation of IFIT-I or STAT1 (Figure 15G). Knockdown of RIG-I expression resulted in a strong reduction of 5'ppp-SEQ ID NO: 1 -induced IFIT-I upregulation, whereas the 5'ppp-SEQ ID NO: 1 -induced upregulation of IFIT-I was not

affected by STAT-1 depletion. siRNA-mediated knockdown of STING also did not block the 5'ppp-SEQ ID NO: 1-induced upregulation of STAT1, indicating that STAT1 and STING are dispensable for the response to 5'ppp-SEQ ID NO: 1, whereas RIG-I is required.

Example 13 - Postinfection treatment with 5'ppp-SEQ ID NO: 1 inhibits CHIKV replication and stimulates the RIG-I pathway in both uninfected and CHIKV-infected cells

To explore the antiviral potential of 5'ppp-SEQ ID NO: 1 against CHIKV, MRC-5 cells were first infected with CHIKV LS3-GFP at an MOI of 0.1, followed by transfection with 5'ppp-SEQ ID NO: 1 (1 ng/ml) or control RNA at several time points postinfection. Measurement of EGFP expression by the reporter virus in infected MRC-5 cells that were fixed at 24 h p.i. indicated that treatment with 5'ppp-SEQ ID NO: 1 at 1 or 3 h p.i. reduced reporter gene expression to less than 20% of that in untreated infected control cells (Figure 16A). Even when treatment was initiated as late as 5 h p.i., a more than 50% reduction in EGFP expression was observed (Figure 16A). Transfection of control RNA merely led to a 20% reduction in EGFP reporter gene expression, largely independent of the time of addition. Postinfection treatment of CHIKV-infected cells with 5'ppp-SEQ ID NO: 1 also reduced viral progeny titers at 24 h p.i., depending on the time of addition (Figure 16B). CHIKV titers in the medium of untreated infected cells were 6×10^6 PFU/ml at 24 h p.i., while treatment from 1 h p.i. onward led to a more than 2-log reduction in infectious progeny, i.e., 5×10^4 PFU/ml. When treatment was initiated at 3, 5, or 8 h p.i., CHIKV titers of 2×10^5 , 7×10^5 , and 1×10^6 , respectively, were measured at 24 h p.i. Transfection of CHIKV-infected cells with control RNA resulted in a less than 1-log reduction in infectious progeny titer (Figure 16B).

To assess the activation of the RIG-I signaling pathway in MRC-5 cells after 5'ppp-SEQ ID NO: 1 treatment in the presence or absence of CHIKV infection, the expression levels of STAT1, RIG-I, and IFIT1 were analyzed by immunoblotting (Figure 16C). Both in mock infected and CHIKV-infected cells, transfection of 0.1 ng/ml 5'ppp-SEQ ID NO: 1

induced a strong upregulation of STAT1, RIG-I, and IFIT-I (Figure 16C), an effect that was more pronounced with treatment of 1 or 10 ng/ml of 5'ppp-SEQ ID NO: 1. In contrast, introduction of control RNA had no effect on expression of these proteins. CHIKV infection alone did not lead to increased STAT1, RIG-I, and IFIT1 expression, and CHIKV infection did not inhibit the 5'ppp-SEQ ID NO: 1-induced upregulation of RIG-I or downstream IFN signaling (Figure 16C).

Example 14 – Materials and Methods:

Materials and Methods in this Example are in reference to Examples 8-13 above.

In vitro synthesis of 5'ppp-SEQ ID NO: 1. The sequence of 5'ppp-SEQ ID NO: 1 was derived from the 5' and 3' untranslated regions (UTR) of the VSV genome as described above. In vitro-transcribed RNA was prepared as described above and in Goulet ML et al, PLoS Pathol 9, e1003298 (2013), which is incorporated by reference herein. RNA was prepared using the Ambion MEGAscript T7 kit according to the manufacturer's guidelines (Invitrogen, NY, USA). 5'ppp-SEQ ID NO: 1 was purified using the Qiagen miRNA minikit (Qiagen, Valencia, CA). An RNA with the same sequence but lacking the 5'ppp moiety was purchased from IDT (Integrated DNA Technologies Inc., IA, USA). This RNA generated results identical to those obtained with 5'ppp-SEQ ID NO: 1 that was dephosphorylated enzymatically with calf intestinal alkaline phosphatase (Invitrogen, NY, USA).

Cell culture and transfections. A549 cells were grown in F12K medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and antibiotics. C6/36 insect cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and antibiotics. Lipofectamine RNAiMax (Invitrogen, NY, USA) was used for transfections of 5'ppp-SEQ ID NO: 1 in A549 cells according to the manufacturer's instructions. For short interfering RNA (siRNA) knockdown, A549 cells were transfected with 50 nM (30 pmol) human RIG-I (sc-6180), IFN- α / β R α chain (sc-35637) and β chain (sc-40091), STING (sc-92042), TLR3 (sc-36685), MDA5 (sc-61010), MAVS (sc-75755), interleukin-28R (IL-28R; sc-62497), IL-10R¹ (sc-75331), STAT1 p844/91 (sc-44123), IRF1

(sc-35706), IRF3 (sc-35710), IRF7 (sc-38011), and control siRNA (sc-37007) (Santa Cruz Biotechnology, Dallas, T) using Lipofectamine RNAiMax according to the manufacturer's guidelines.

MRC-5 cells (ATCC CCL-171) were grown in Earle's minimum essential medium (EMEM) supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids (PAA), and antibiotics. For siRNA mediated knockdown of gene expression, MRC-5 cells were transfected with 16.7nM(10 pmol) siRNA using Dharmafect1 (Dharmacon) according to the manufacturer's guidelines. Mouse embryonic fibroblast cells (MEFs) were grown in DMEM with 10% FBS and antibiotics.

Primary cell isolation. Human peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers in a study approved by the institutional review board and by the VGTI-FL Institutional Biosafety Committee (2011-6-JH1). Written informed consent, approved by the VGTI-FL Inc. ethics review board (FWA number 161), was provided and signed by study participants. Research conformed to ethical guidelines established by the ethics committee of the OHSU VGTI and Martin Health System. Briefly, PBMC were isolated from freshly collected blood using Ficoll-Paque plus medium (GE Healthcare Bio, Uppsala, Sweden) per the manufacturer's instructions. Monocytes were then isolated using the negative selection human monocyte enrichment kit (Stem Cell, Vancouver, Canada) per the kit's instructions and used for further experiments. To obtain monocyte-derived dendritic cells (MDDC), monocytes were allowed to adhere to 100-mm dishes for 1 h in serum-free RPMI at 37°C. After adherence, remaining platelets and nonadherent cells were removed by two washes with serum-free RPMI. The cells were differentiated into MDDC by culturing for 7 days in Mo-DC differentiation medium (Miltenyi Biotec, Auburn, GA). Medium was replenished after 3 days of differentiation.

Virus production, quantification, and infection. Confluent monolayers of C6/36 insect cells were infected with DENV serotype 2 strain New Guinea C (DENV NGC) at a multiplicity of infection (MOI) of 0.5. Virus was allowed to adsorb for 1 h at 28°C in a

minimal volume of serum-free DMEM. After adsorption, the monolayer was washed once with serum free medium and covered with DMEM containing 2% FBS. After 7 days of infection, medium was harvested, cleared by centrifugation (500 x g, 5 min), and concentrated down by centrifugation (2,000 x g, 8 min) through a 15-ml Millipore Amicon centrifugal filter unit (Millipore, Billerica, MA). The virus was concentrated by ultracentrifugation on a sucrose density gradient (20% sucrose cushion) using a Sorvall WX 100 ultracentrifuge (ThermoScientific, Rockford, IL) for 2 h at 134,000 x g and 10°C with the brake turned off. Concentrated virus was then washed to remove sucrose using a 15-ml Amicon tube. After 2 washes, the virus was resuspended in DMEM plus 0.1% bovine serum albumin (BSA) and stored at -80°C. Titers of DENV stocks were determined by fluorescence activated cell sorting (FACS), infecting Vero cells with 10-fold serial dilutions of the stock, and then immunofluorescence staining of intracellular DENV E protein at 24 h postinfection (p.i.). Titers were expressed as IU/ml. DENV titers in cell culture supernatants from 5'ppp-SEQ ID NO: 1 -treated and control cells were determined by plaque assay on confluent Vero cells. Cells in 6-well clusters were incubated with 10-fold serial dilutions of the sample in a total volume of 500 µl of DMEM without serum. After 1 h of infection, the inoculum was removed and cells were overlaid with 3ml of 2% agarose in complete DMEM. The cells were fixed and stained, and plaques were counted 5 days postinfection.

In infection experiments, A549 cells, monocytes, or MDDC were infected in a small volume of medium without FBS for 1 h at 37°C and then incubated with complete medium for 24 to 72 h prior to analysis. All procedures with live DENV were performed in a biosafety level 2 facility at the Vaccine and Gene Therapy Institute–Florida.

Chikungunya virus (CHIKV) strain LS3 and enhanced green fluorescent protein (EGFP)-expressing reporter virus CHIKV LS3-GFP have been described (Scholte FE et al, PLoS One 8, e71047 (2013); incorporated by reference herein). Virus production, titration, and infection were performed essentially as described in the art. Working stocks of CHIKV were routinely produced in Vero E6 cells at 37°C, and infections were

performed in EMEM with 25 mM HEPES (Lonza) supplemented with 2% fetal calf serum (FCS), L-glutamine, and antibiotics. After 1 h, the inoculum was replaced with fresh culture medium. All procedures with live CHIKV were performed in a biosafety level 3 facility at the Leiden University Medical Center.

Flow cytometry analysis. The percentage of cells infected with DENV was determined by standard intracellular staining (ICS) with a mouse IgG2a monoclonal antibody (MAb) specific for DENV-E protein (clone 4G2), followed by staining with a secondary anti-mouse antibody coupled to phycoerythrin (PE) (BioLegend, San Diego, CA). Cells were analyzed on an LSRII flow cytometer (Becton, Dickinson, New Jersey, USA). Calculations as well as population analyses were done using FACS Diva software.

Cell viability analysis. Cell surface expression of phosphatidylserine was measured using an allophycocyanin (APC)-conjugated annexin V antibody, as recommended by the manufacturer (BioLegend, San Diego, CA). Briefly, specific annexin V binding was achieved by incubating A549 cells in annexin V binding buffer (Becton, Dickinson, NJ, USA) containing a saturating concentration of APC-annexin V antibody and 7-aminoactinomycin D (7-AAD) (Becton, Dickinson, New Jersey, USA) for 15 min in the dark. APC-annexin V and 7-AAD binding to the cells was analyzed by flow cytometry, as described previously, using an LSRII flow cytometer and FACS Diva software. Alternatively, the viability of siRNA or 5'ppp-SEQ ID NO: 1-transfected cells was assessed using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega). Absorbance was measured using a Berthold Mithras LB 940 96-well plate reader.

Protein extraction and immunoblot analysis. DENV-infected cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8, 1% sodium deoxycholate, 1% NP-40, 5 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate), and the insoluble fraction was removed by centrifugation at 17,000 × g for 15 min (4°C). Protein concentration was determined using the Pierce bicinchoninic (BCA) protein assay kit (Thermo Scientific, Rockford, IL). Protein extracts were resolved by SDS-PAGE on 4 to 20% acrylamide Mini-

Protean TGX precast gels (Bio-Rad, Hercules, CA) in a 1× Tris-glycine-SDS buffer (Bio-Rad, Hercules, CA). Proteins were electrophoretically transferred to an Immobilon-PSQ polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) for 1 h at 100 V in a buffer containing 30 mM Tris, 200 mM glycine, and 20% methanol. Membranes were blocked for 1 h at room temperature in Odyssey blocking buffer (Odyssey, USA) and then probed with the following primary antibodies: anti-IRF1 (Santa Cruz Biotechnology, Dallas, TX), anti-pIRF3 at Ser 396 (EMD Millipore, MA, USA), anti-IRF3 (IBL, Japan), anti-IRF7 (Cell Signaling, MA, USA), anti-RIG-I (EMD Millipore, MA, USA), anti-IFIT1 (Thermo Fisher Scientific, Rockford, IL, USA), anti-ISG15 (Cell Signaling Technology, Danvers, MA), anti-pSTAT1 at Tyr701 (Cell Signaling, MA, USA), anti-STAT1 (Cell Signaling, MA, USA), anti-STING (Novus Biologicals, Littleton, CO), anti-DENV (Santa Cruz Biotechnology, USA), and anti- β -actin (Odyssey, USA). Antibody signals were detected by immunofluorescence using the IRDye 800CW and IRDye 680RD secondary antibodies (Odyssey, USA) and the LiCor imager (Odyssey, USA). Protein expression levels were determined and normalized to β -actin using ImageJ software (National Institutes of Health, Bethesda, MD).

CHIKV-infected cells were lysed and proteins were analyzed by Western blotting. CHIKV proteins were detected with rabbit antisera against nsP1 (a generous gift of Andres Merits, University of Tartu, Estonia) and E2 (Aguirre S, PLoS Pathog 8, 31002934 (2012); incorporated by reference herein). Mouse monoclonal antibodies against β -actin (Sigma), the transferrin receptor (Zymed), cyclophilin A (Abcam), and cyclophilin B (Abcam) were used for detection of loading controls. Biotin-conjugated swine α -rabbit (Dako), goat α -mouse (Dako), and Cy3-conjugated mouse α -biotin (Jackson) were used for fluorescent detection of the primary antibodies with a Typhoon-9410 scanner (GE Healthcare).

RT-qPCR. Total RNA was isolated from cells using an RNeasy kit (Qiagen, Valencia, CA) per the manufacturer's instructions. RNA was reverse transcribed using the SuperScript VILO cDNA synthesis kit according to the manufacturer's instructions

(Invitrogen, Carlsbad, CA). PCR primers were designed using Roche's Universal Probe Library Assay Design Center (Roche). Quantitative reverse transcription-PCR (RTqPCR) was performed on a LightCycler 480 system using LightCycler 480 probes master (Roche, Penzberg, Germany). All data are presented as a relative quantification with efficiency correction based on the relative expression of target gene versus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the invariant control. The N-fold differential mRNA expression of genes in samples was expressed as $2^{\Delta\Delta CT}$. Primers used are described in the Sequence Listing submitted with this application.

RNA isolation, denaturing agarose electrophoresis, and in-gel hybridization.

CHIKV RNA isolation and analysis were performed essentially as described in the art. Briefly, total RNA was isolated by lysis in 20 mM Tris-HCl (pH 7.4), 100 mM LiCl, 2 mM EDTA, 5 mM dithiothreitol (DTT), 5% (wt/vol) lithium dodecyl sulfate, and 100 µg/ml proteinase K. After acid phenol (Ambion) extraction, RNA was precipitated with isopropanol, washed with 75% ethanol, and dissolved in 1 mM sodium citrate (pH 6.4). RNA samples were separated in 1.5% denaturing formaldehyde-agarose gels using the morpholine propanesulfonic acid (MOPS) buffer system. RNA molecules were detected by direct hybridization of the dried gel with ³²P-labeled oligonucleotides. CHIKV genomic and subgenomic RNAs (sgRNAs) were visualized with probe CHIKV-hyb4 and negative-stranded RNA was detected with probe CHIKV-hyb2. Probes (10 pmol) were labeled with 10 µCi [γ -³²P]ATP (PerkinElmer). Prehybridization (1 h) and hybridization (overnight) were done at 55°C in 5x SSPE (0.9 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, pH 7.4), 5x Denhardt's solution, 0.05% SDS, and 0.1 mg/ml homomix I. Storage Phosphor screens were exposed to hybridized gels and scanned with a Typhoon-9410 scanner (GE Healthcare), and data were quantified with Quantity One v4.5.1 (Bio-Rad).

Statistical analysis. Values were expressed as the means $\pm$ standard errors of the means (SEM), and statistical analysis was performed with Microsoft Excel using an unpaired, two-tailed Student's t test to determine significance. Differences were considered significant at $P < 0.05$

CLAIMS

1. A compound comprising
an oligoribonucleotide comprising a nucleic acid sequence of SEQ ID NO: 1; and
a triphosphate group covalently attached to the 5' end of the
oligoribonucleotide.
2. The compound of claim 1 wherein the oligoribonucleotide consists of SEQ ID NO:
1.
3. The compound of claim 1 wherein the oligoribonucleotide comprises a modified
ribonucleotide.
4. The compound of claim 3 wherein the modified oligoribonucleotide comprises a
ribonucleotide comprising 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-
methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl.
5. The compound of claim 3 wherein the modified ribonucleotide comprises a
locked nucleic acid.
6. The compound of claim 5 wherein the locked nucleic acid is 2'-O, 4'-C-
methylene-(D-ribofuranosyl)nucleotide, 2'-O-(2-methoxyethyl) (MOE) nucleotide, 2'-
methyl-thio-ethyl nucleotide, 2'-deoxy-2'-fluoro (2'F) nucleotide, 2'-deoxy-2'-chloro (2Cl)
nucleotide, or 2'-azido nucleotide.
7. The compound of claim 3 wherein the modified nucleotide comprises a G-clamp
nucleotide.

8. The compound of claim 3 wherein the modified nucleotide comprises a nucleotide base analog.
9. The compound of claim 8 wherein the nucleotide base analog comprises C-phenyl, C-naphthyl, inosine, azole carboxamide, or nitroazole.
10. The compound of claim 9 wherein the moiety is nitroazole and is 3-nitropyrrole, 4-nitroindole, 5-nitroindole, or 6-nitroindole.
11. The compound of claim 1 comprising a 3' terminal cap moiety.
12. The compound of claim 11 wherein the terminal cap moiety is an inverted deoxy abasic residue, a glyceryl modification, a 4', 5'-methylene nucleotide, a 1-(β -D-erythrofuransyl) nucleotide, a 4'-thio nucleotides, carbocyclic nucleotide, a 1, 5-anhydrohexitol nucleotide, an L-nucleotide, an α -nucleotide, a modified base nucleotide, a threo pentofuransyl nucleotide, an acyclic 3',4'-seco nucleotide, an acyclic 3,4-dihydroxybutyl nucleotide, an acyclic 3,5-dihydroxypentyl nucleotide, a 3'-3'-inverted nucleotide moiety, a 3'-3'-inverted abasic moiety, a 3'-2'-inverted nucleotide moiety, a 3'-2'-inverted abasic moiety, a 5'-5'-inverted nucleotide moiety, a 5'-5'-inverted abasic moiety, a 3'-5'-inverted deoxy abasic moiety, a 5'-amino-alkyl phosphate, a 1,3-diamino-2-propyl phosphate, a 3-aminopropyl phosphate, a 6-aminohexyl phosphate, a 1, 2-aminododecyl phosphate, a hydroxypropyl phosphate, a 1,4-butanediol phosphate, a 3'-phosphoramidate, a 5'-phosphoramidate, a hexylphosphate, an aminohexyl phosphate, a 3'-phosphate, a 5'-amino, 3'-phosphorothioate, a 5'-phosphorothioate, a phosphorodithioate, a bridging methylphosphonate, a non-bridging methylphosphonate, or a 5'-mercapto group.

13. The compound of claim 1 wherein the oligoribonucleotide comprises a phosphate backbone modification.
14. The compound of claim 13 wherein the phosphate backbone modification is a phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, or alkylsilyl substitution.
15. The compound of claim 1 further comprising a conjugate attached to the oligoribonucleotide.
16. The compound of claim 15 wherein the conjugate is attached to the 3' end of the oligoribonucleotide.
17. A pharmaceutical composition comprising a therapeutically effective amount of any of the compounds of claims 1-16 and a pharmaceutically acceptable carrier, wherein the pharmaceutically acceptable carrier acts as a transfection reagent.
18. The pharmaceutical composition of claim 17 wherein the pharmaceutically acceptable carrier comprises a lipid based carrier, a polymer based carrier, a cyclodextrin based carrier, or a protein based carriers.
19. The pharmaceutical composition of claim 18 wherein the pharmaceutically acceptable carrier is a lipid based carrier comprising a stabilized nucleic acid-lipid particle, a cationic lipid, a liposome nucleic acid complex, a liposome, a micelle, or a virosome.

20. The pharmaceutical composition of any of claims 17-19 for use in treating a viral infection.

21. The pharmaceutical composition of claim 20 wherein the viral infection is a vesicular stomatitis virus infection, a dengue virus infection, a vaccinia virus infection, a human immunodeficiency virus infection, a chikungunya virus infection, or an influenza virus infection.

Figure 1A

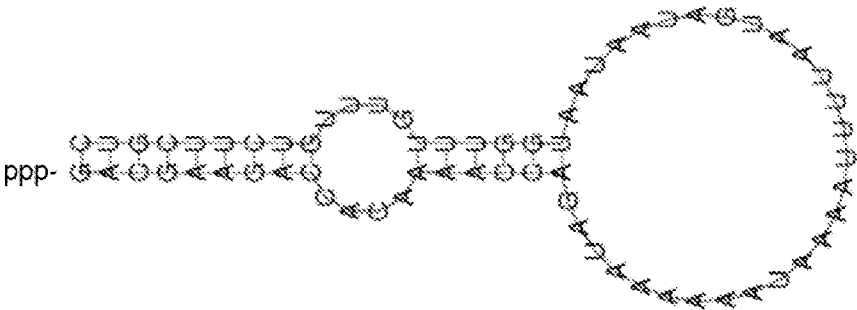


Figure 1B

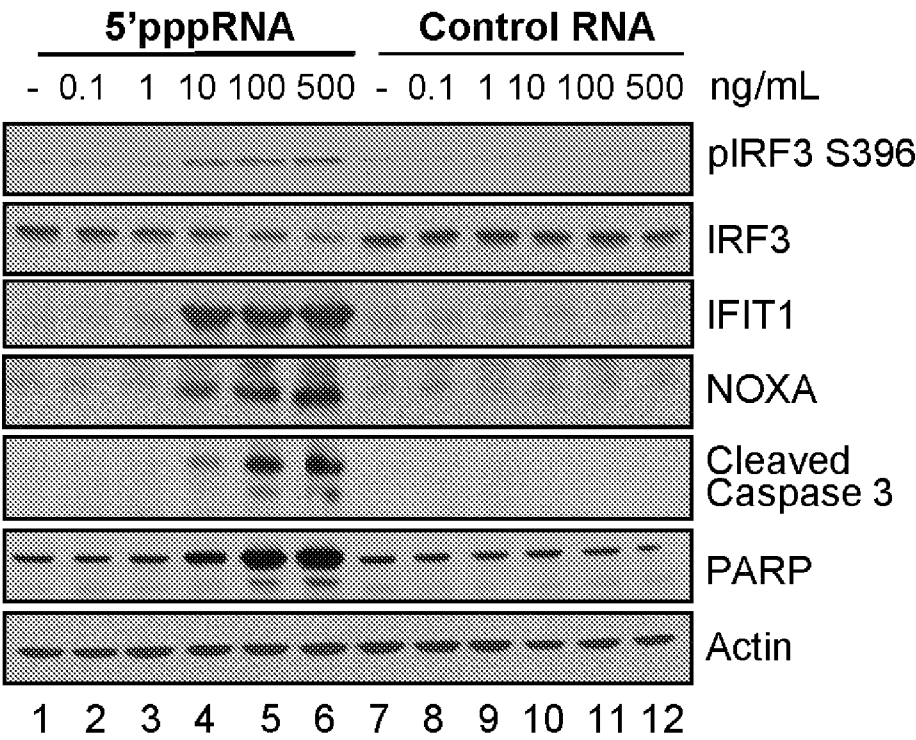


Figure 1C

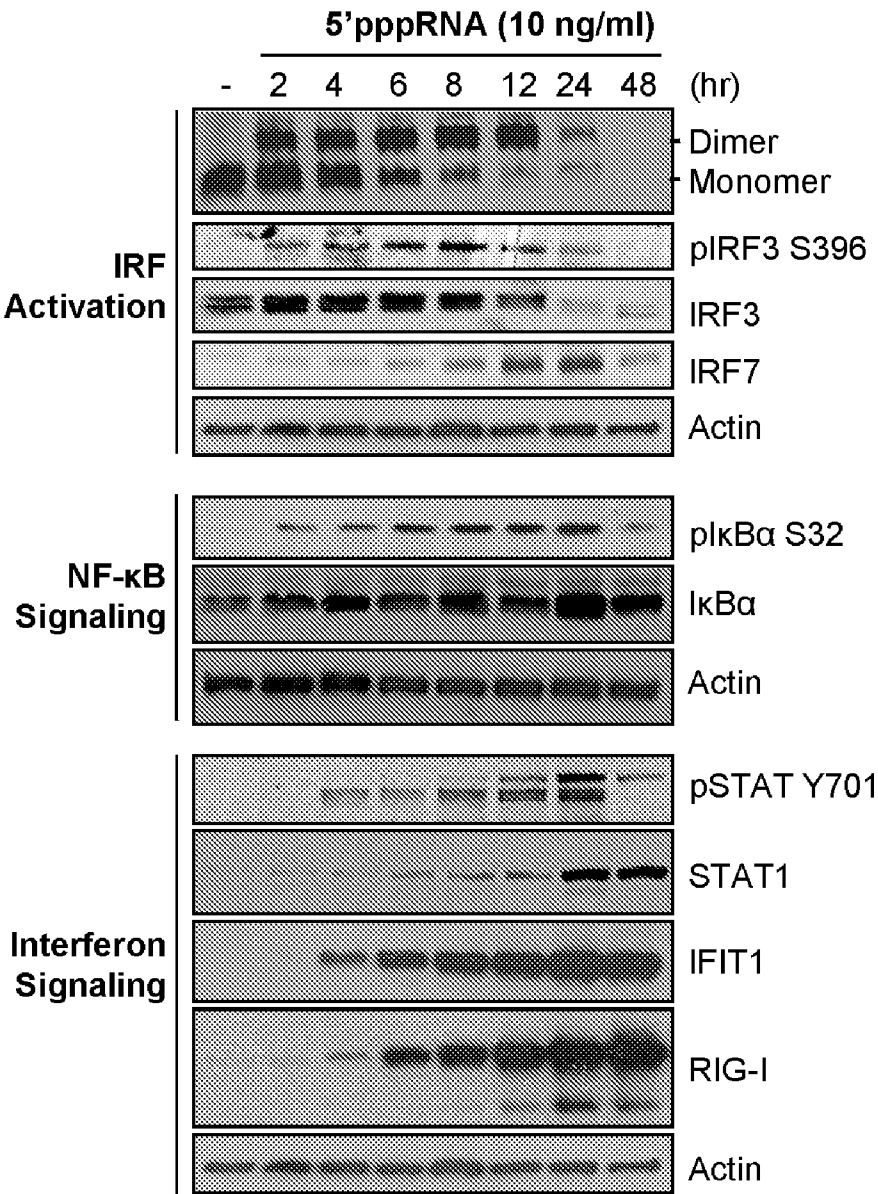


Figure 1D

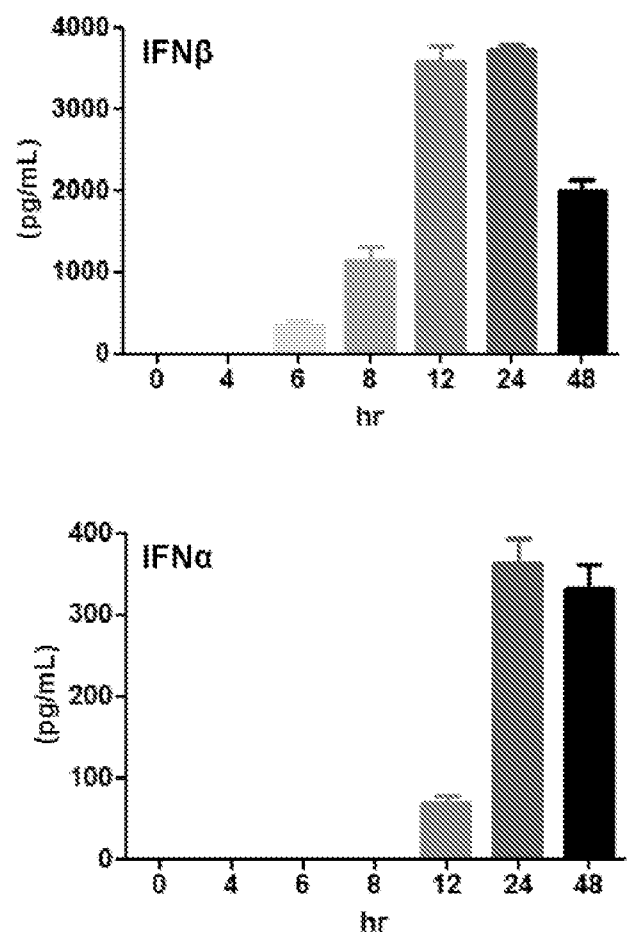


Figure 2A

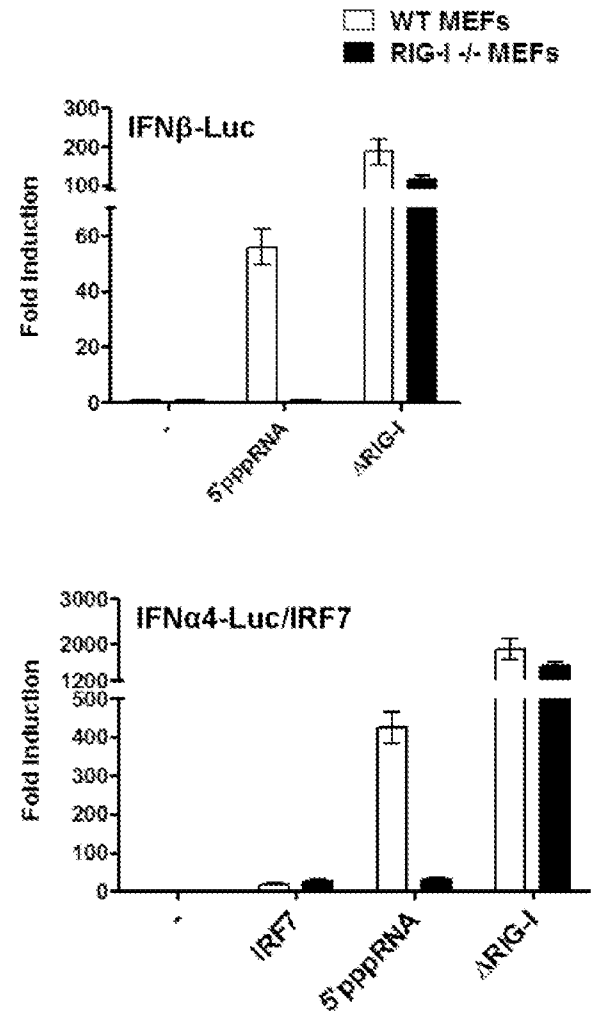


Figure 2B

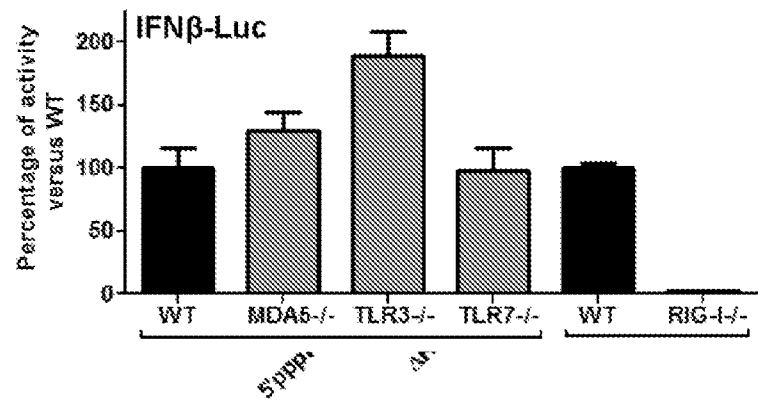


Figure 2C

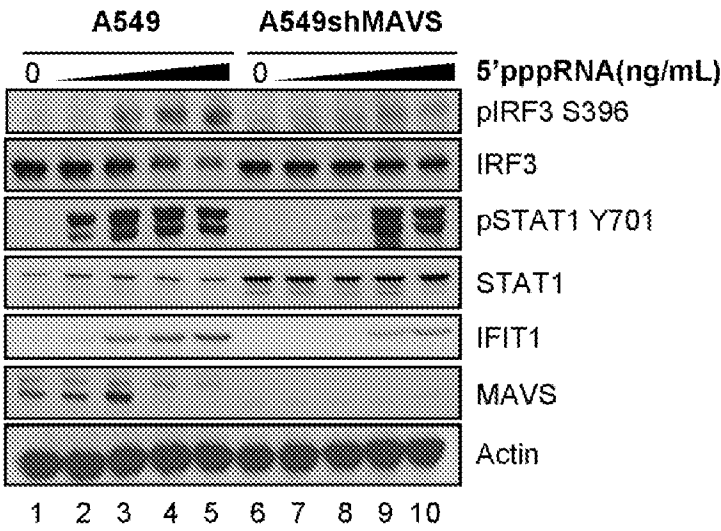


Figure 2D

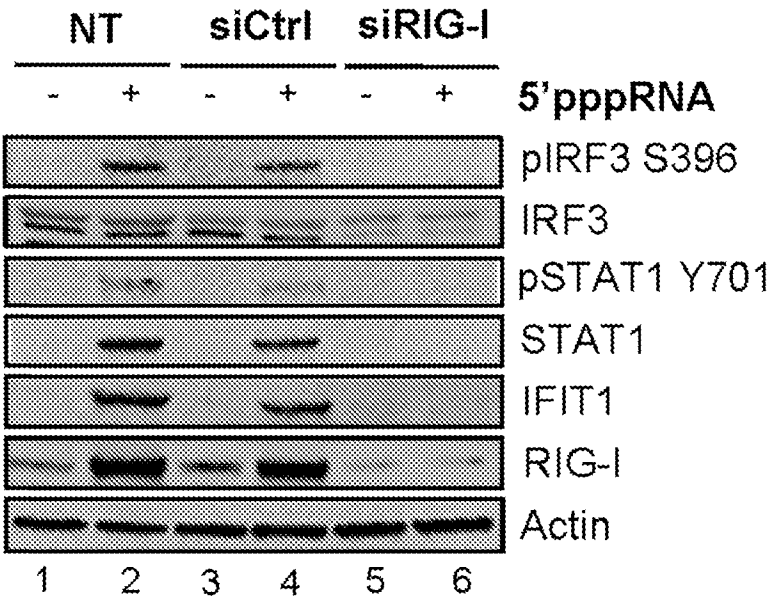


Figure 3A

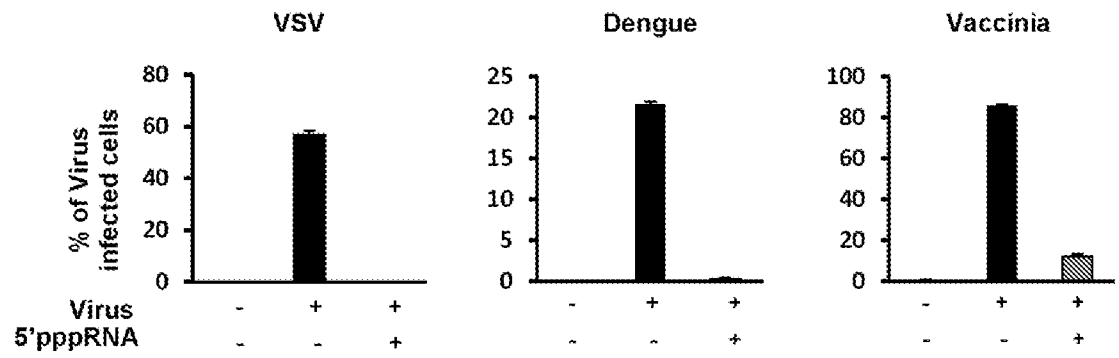


Figure 3B

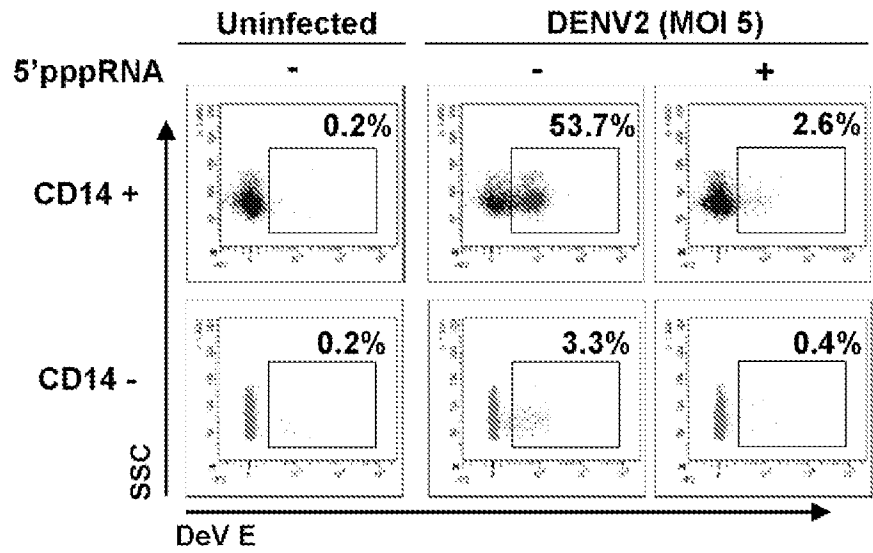


Figure 3C

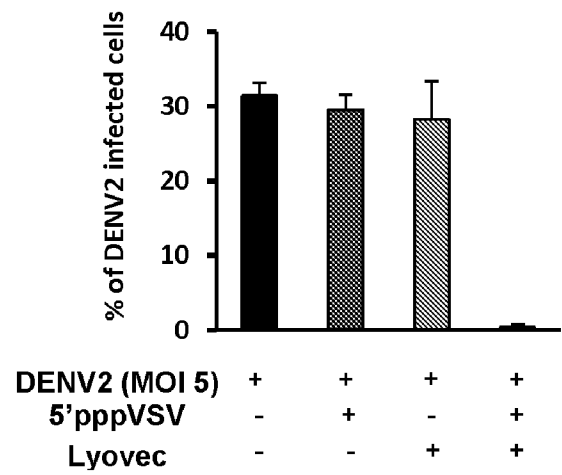


Figure 3D

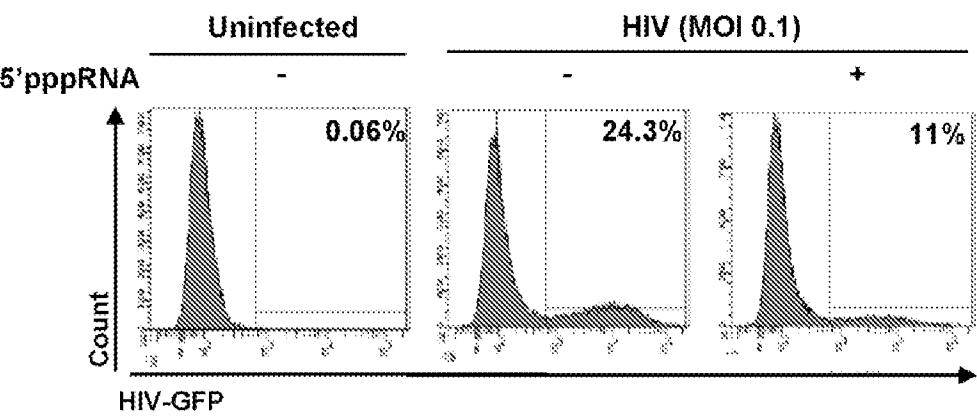


Figure 3E

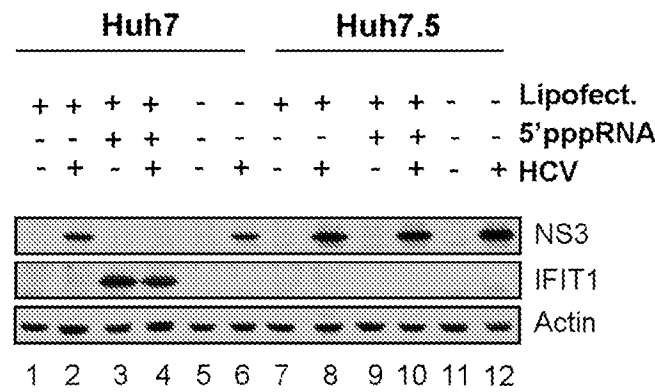


Figure 4A

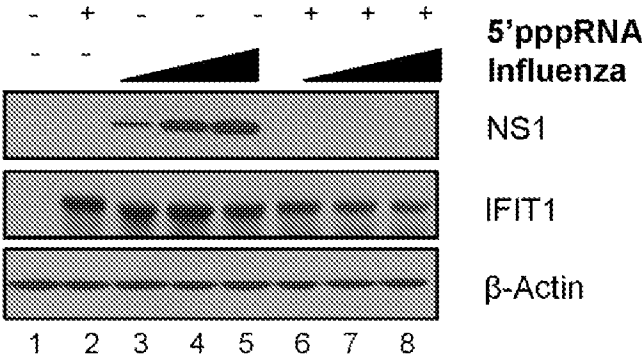


Figure 4B

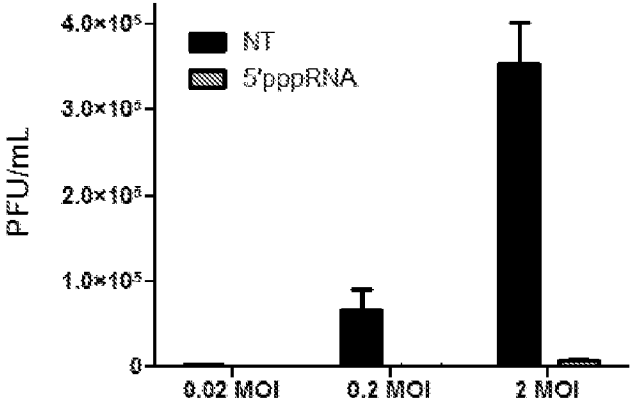


Figure 4C

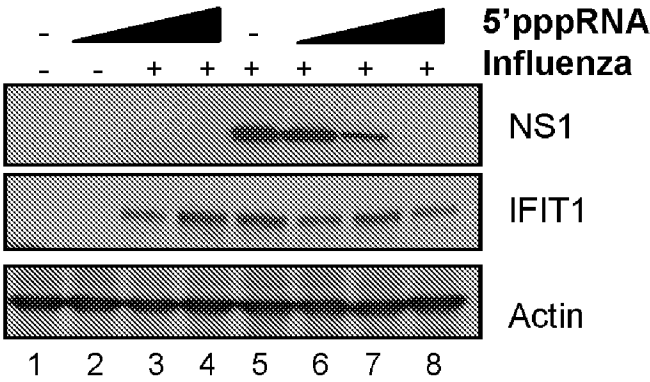


Figure 4D

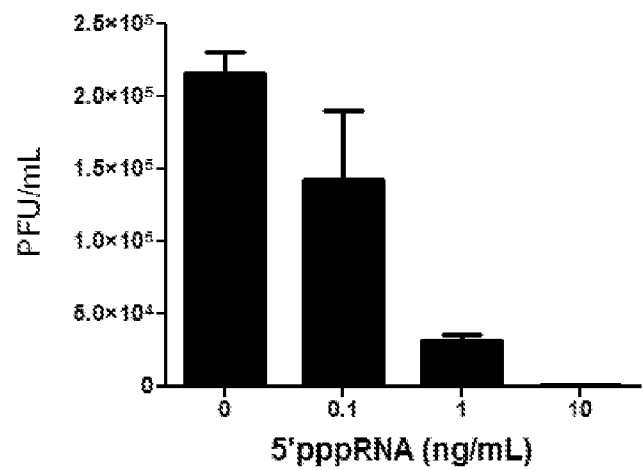


Figure 4E

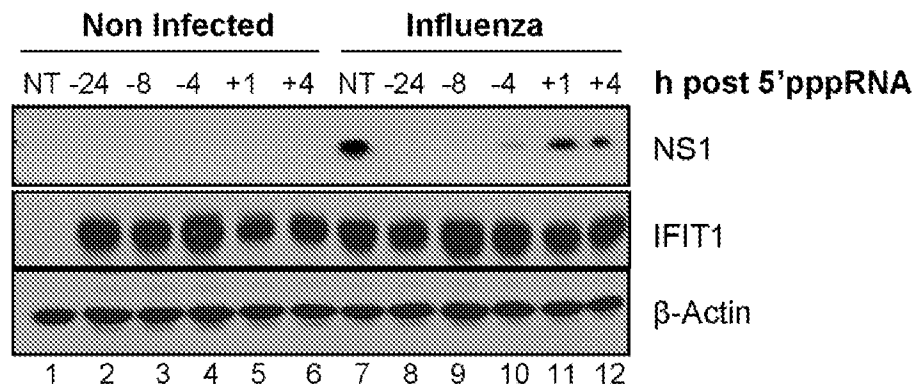


Figure 4F

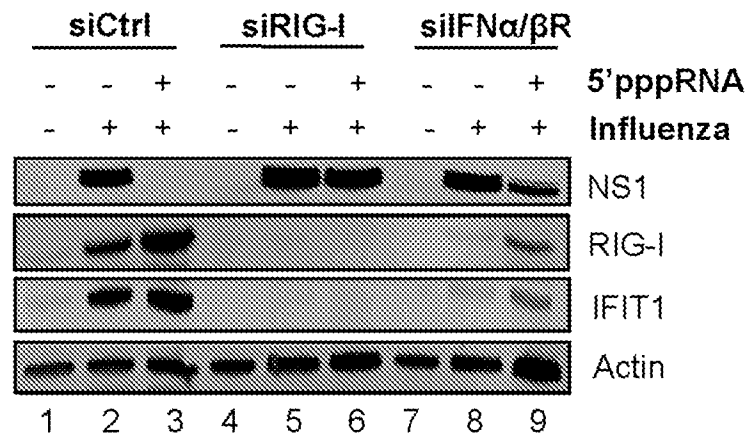


Figure 4G

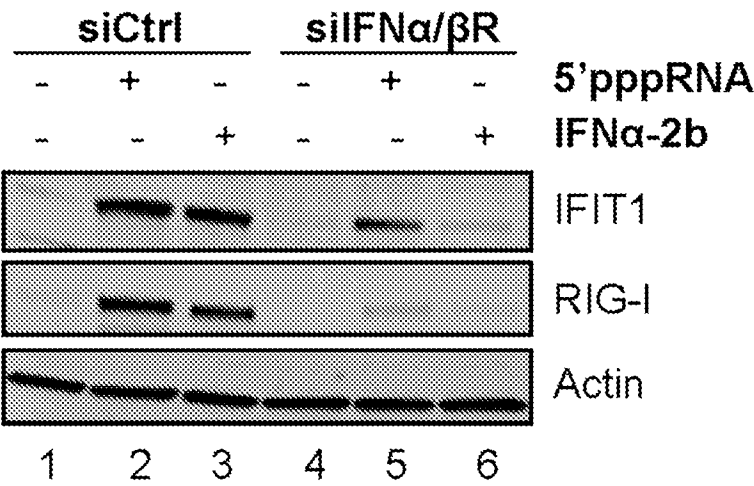


Figure 5A

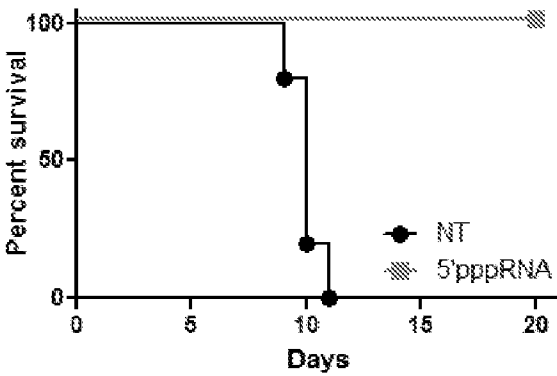


Figure 5B

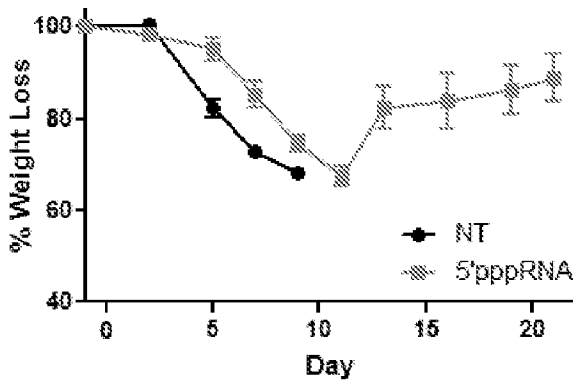


Figure 5C

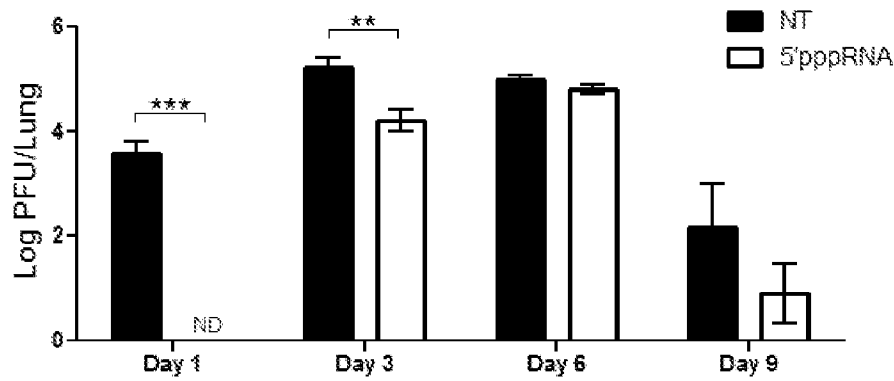


Figure 5D

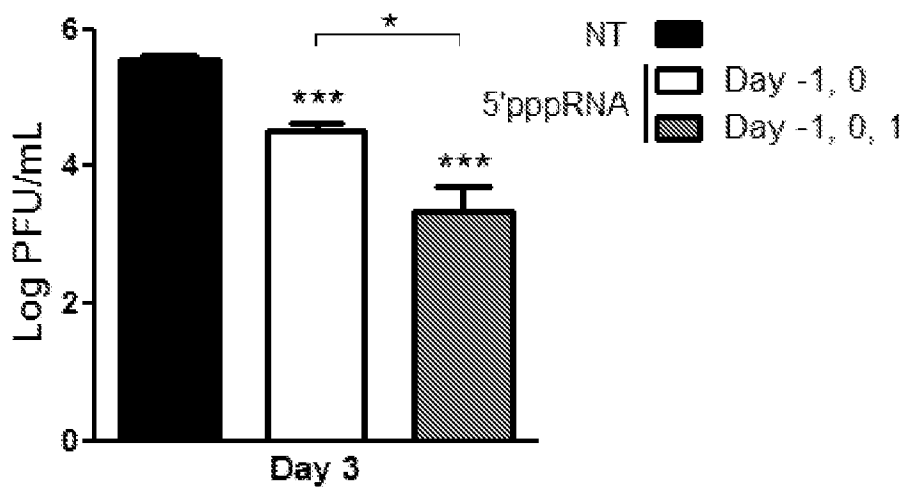


Figure 5E

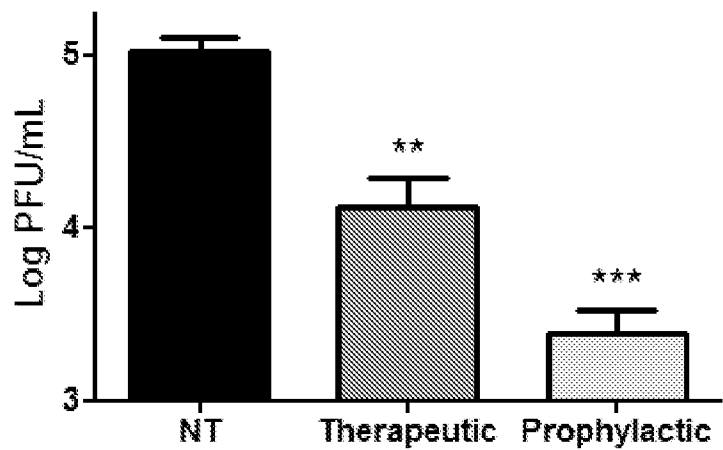


Figure 5F

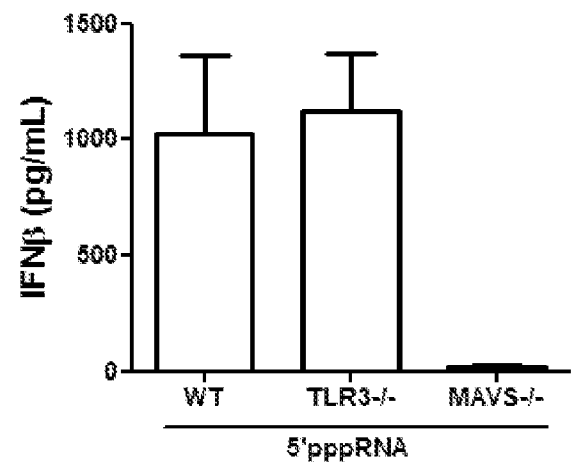


Figure 5G

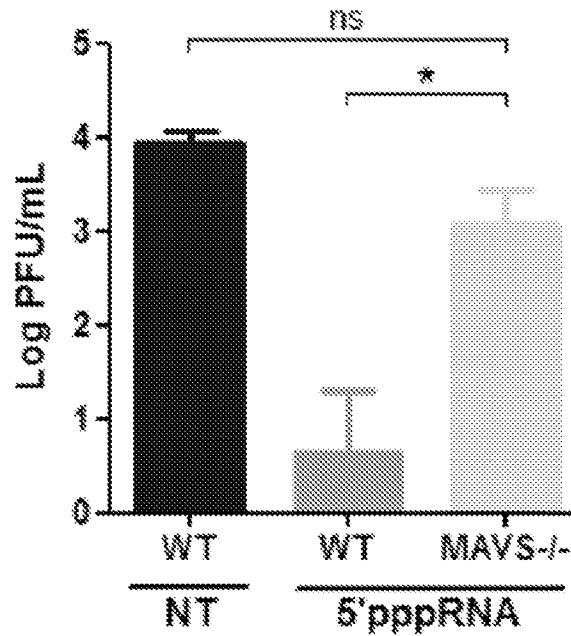


Figure 5H

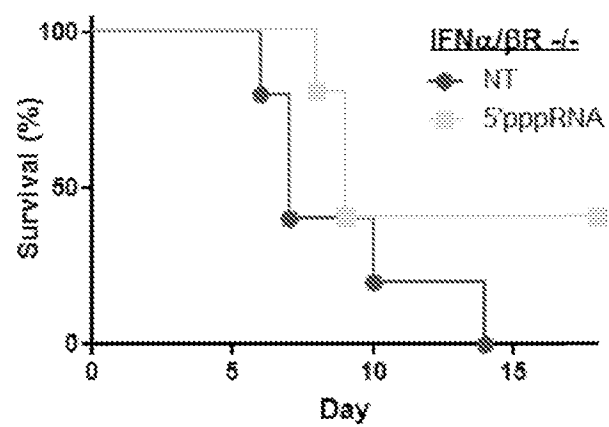


Figure 5I

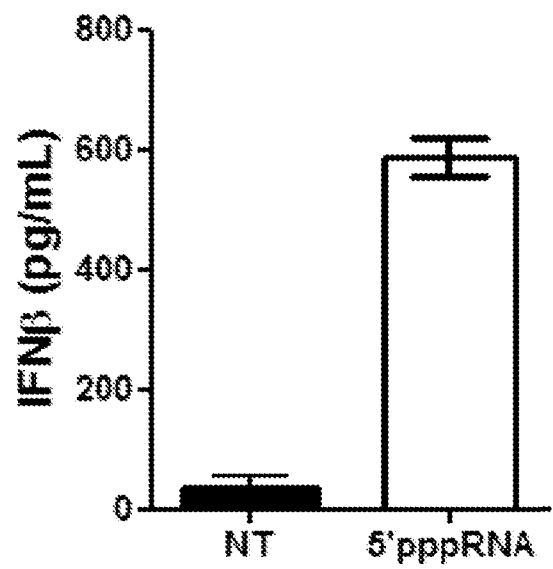


Figure 6A

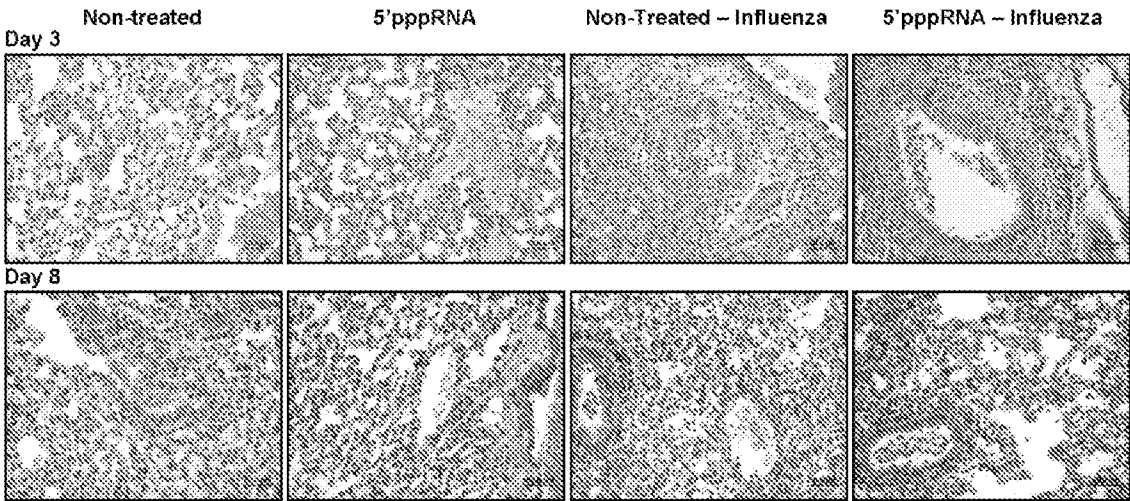


Figure 6B

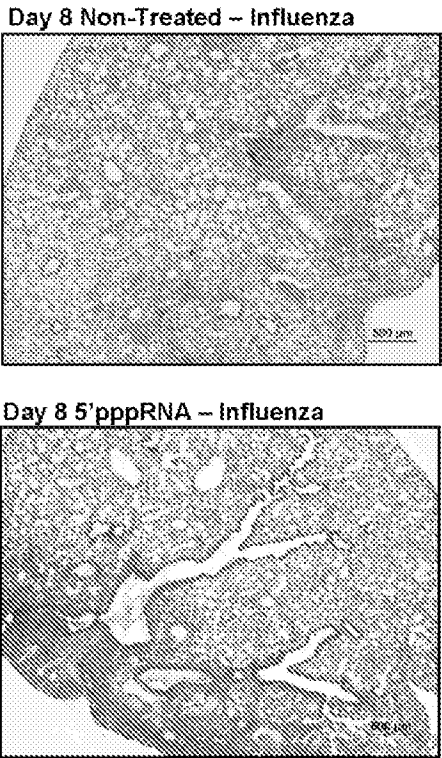


Figure 6C

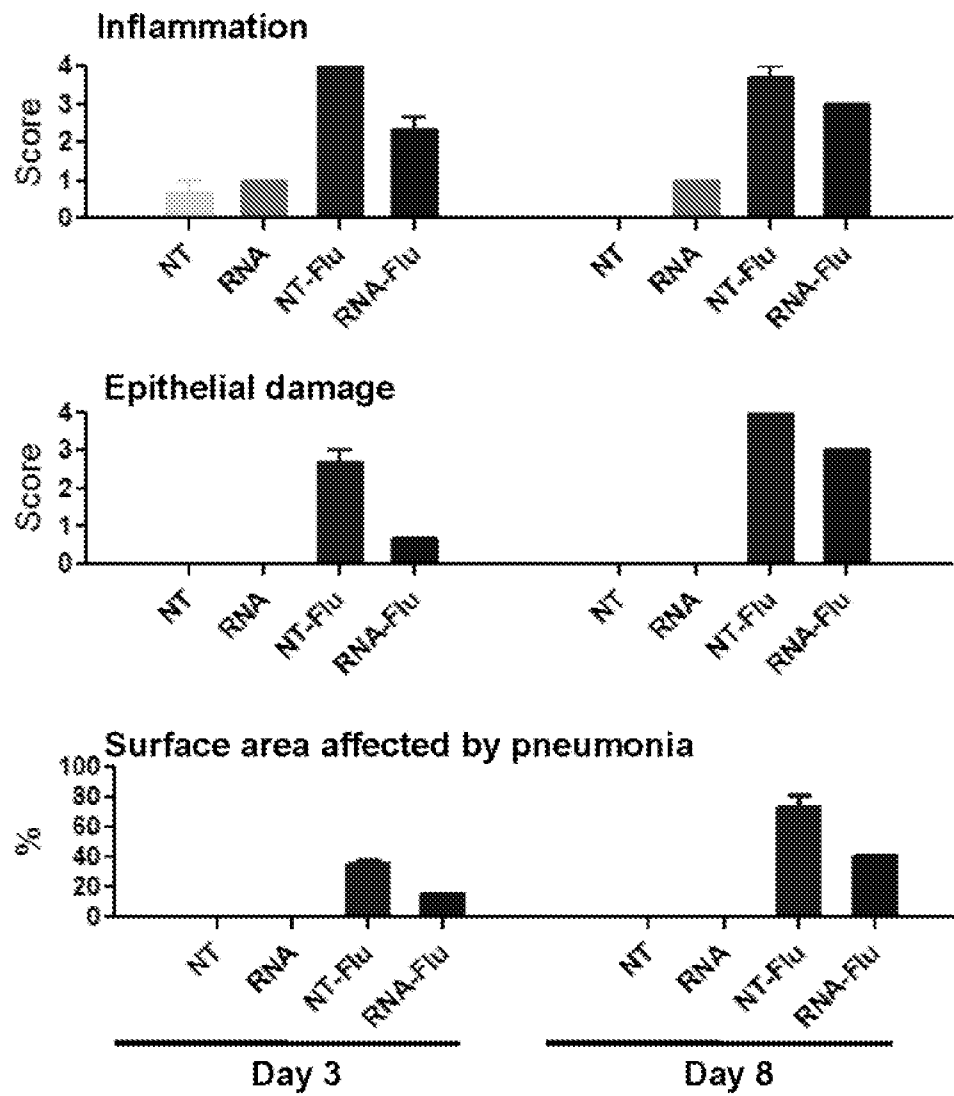


Figure 8A

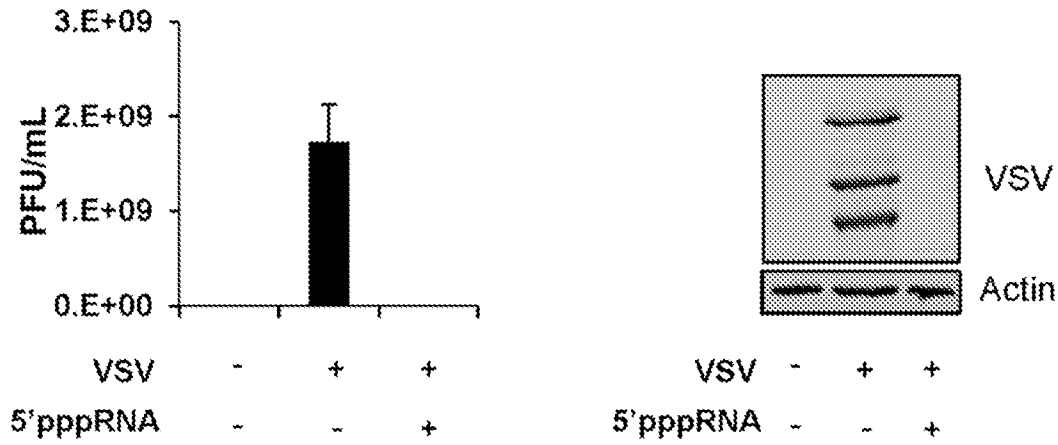


Figure 8B

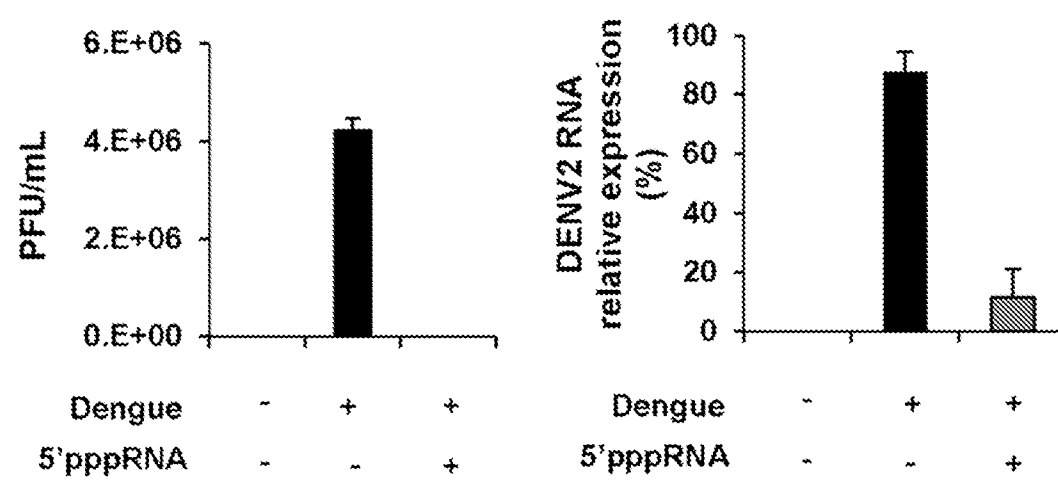


Figure 9A

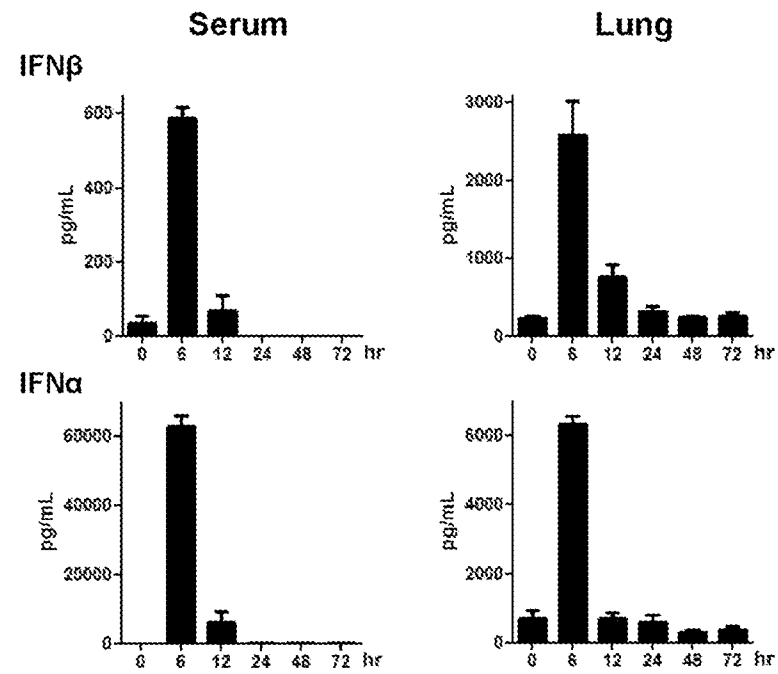


Figure 9B

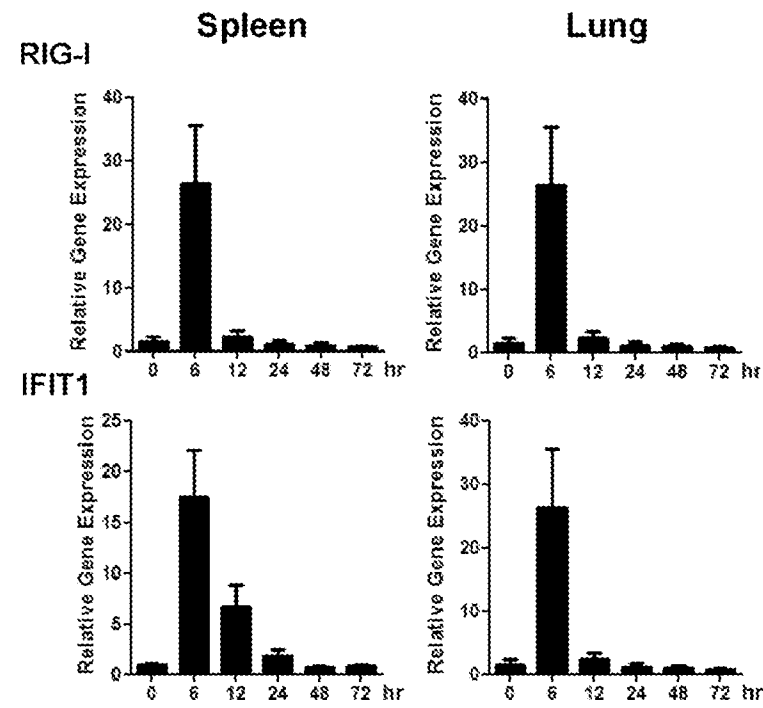


Figure 9C

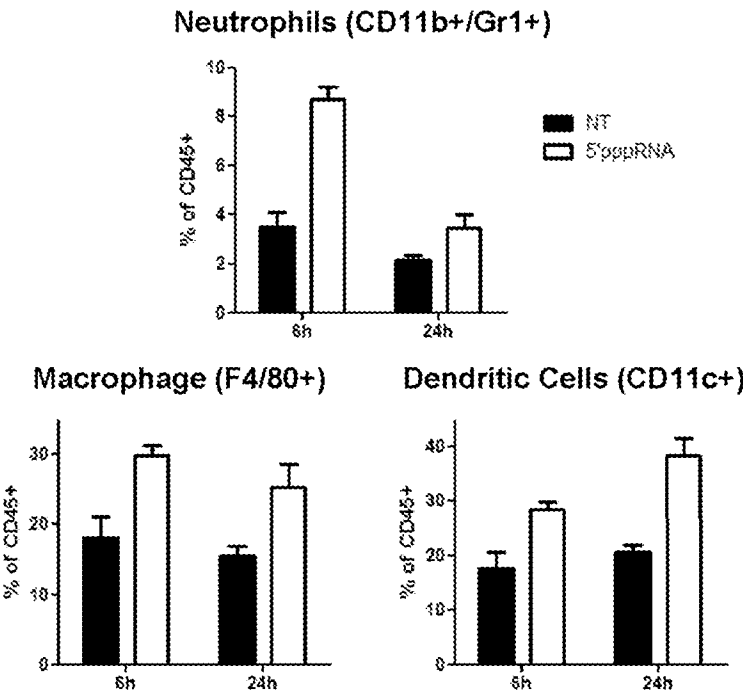


Figure 9D

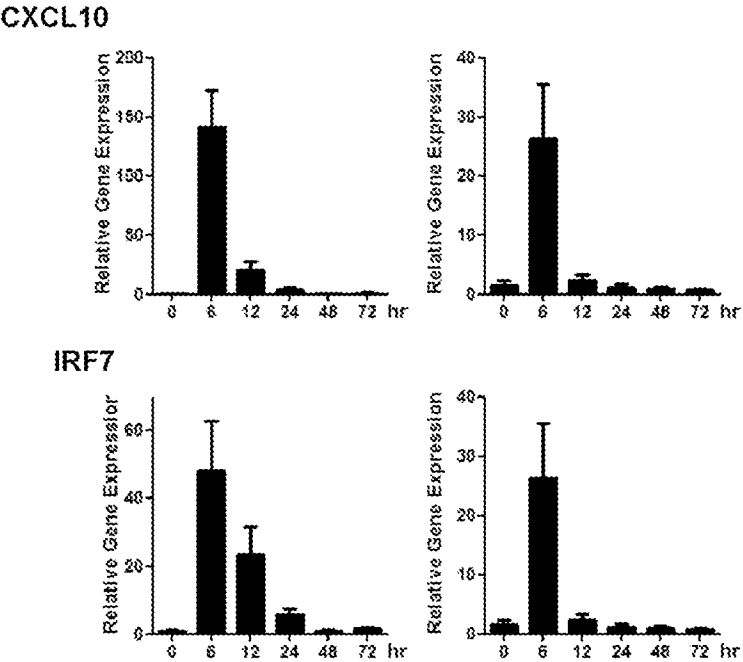


Figure 10A

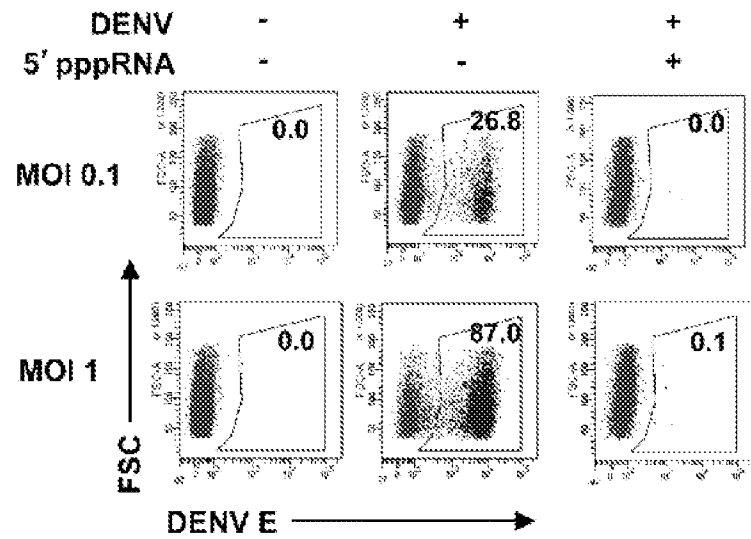


Figure 10B

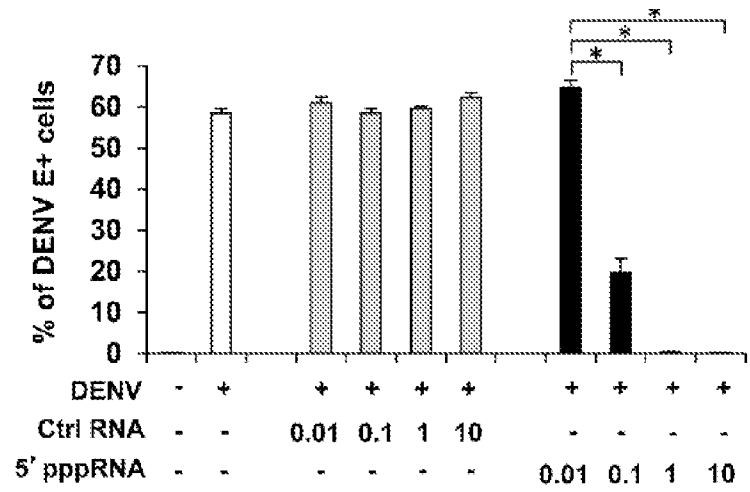


Figure 10C

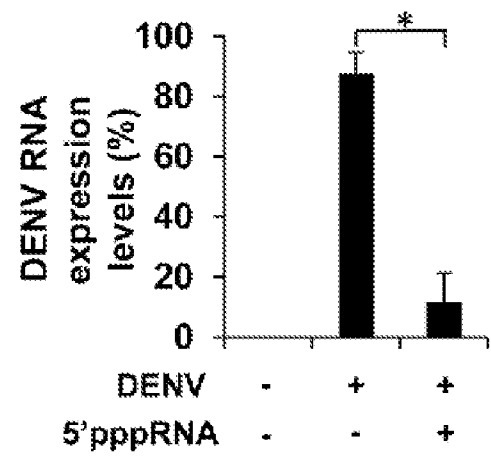


Figure 10D

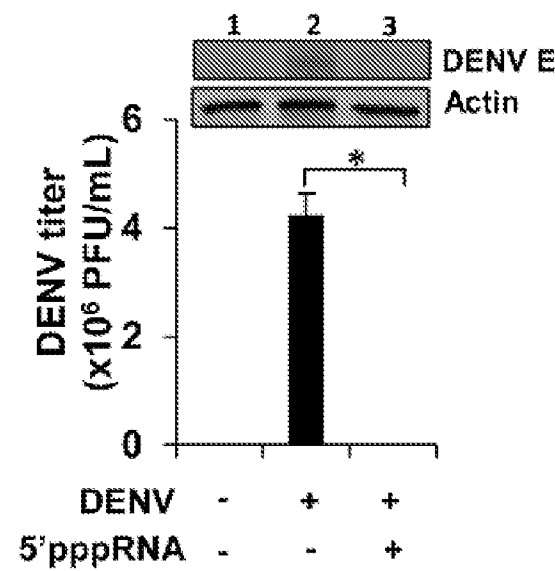


Figure 10E

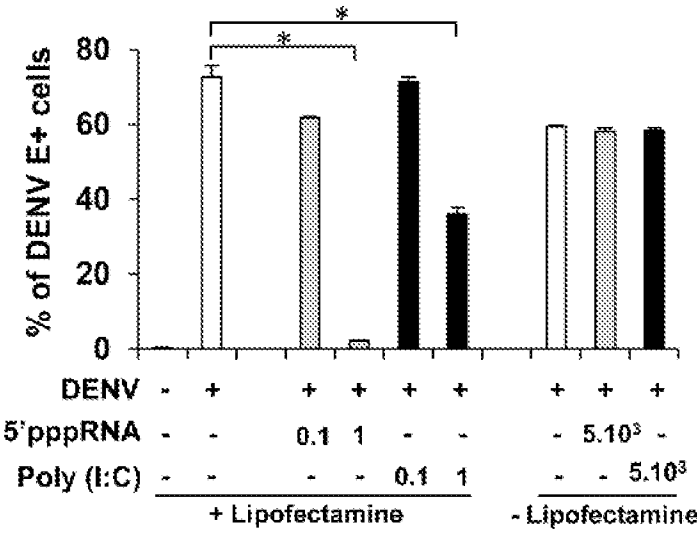


Figure 10F

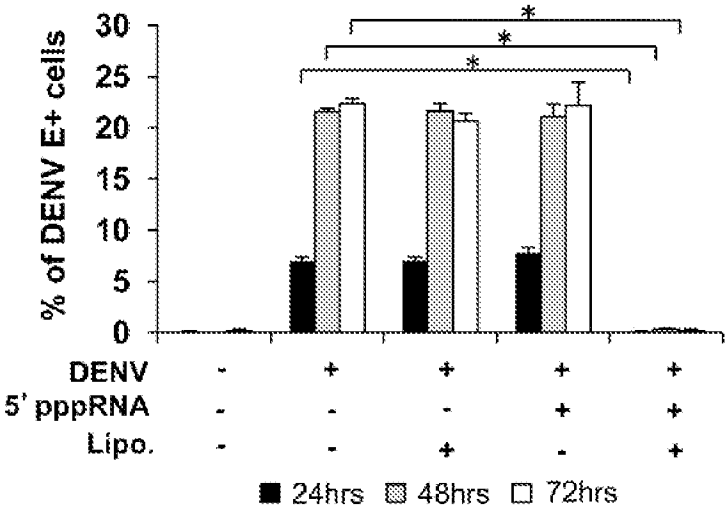


Figure 10G

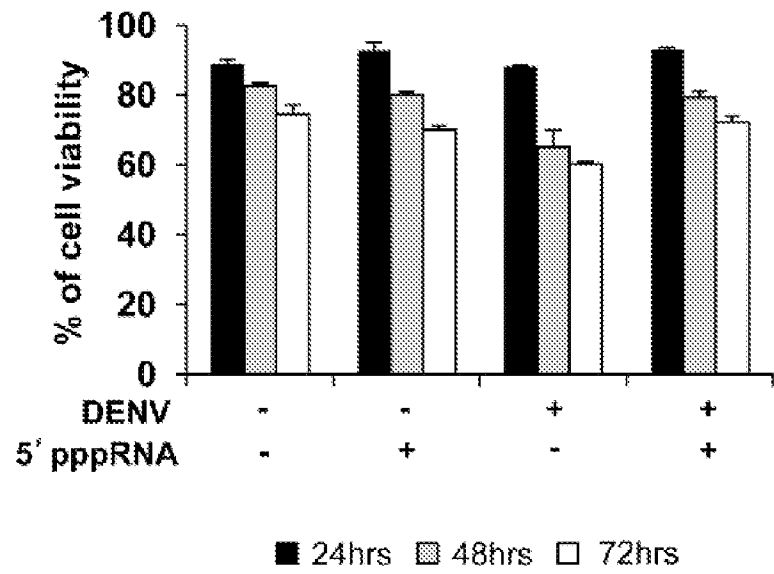


Figure 11A

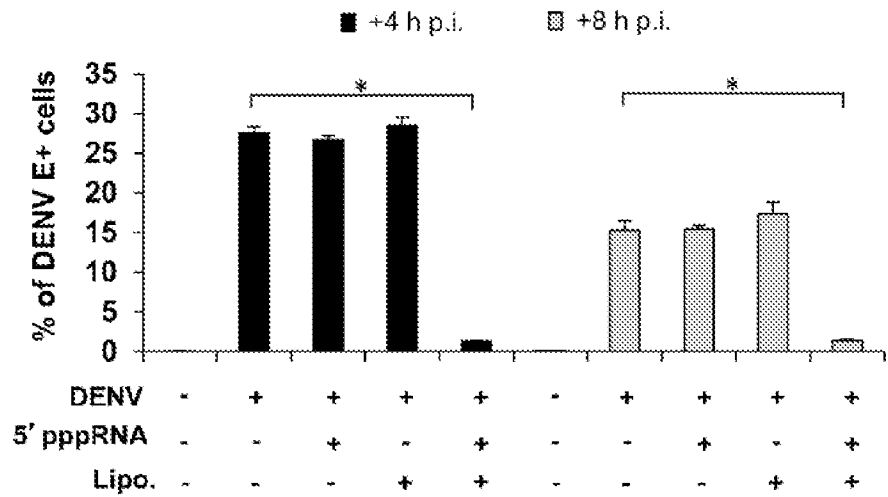


Figure 11B

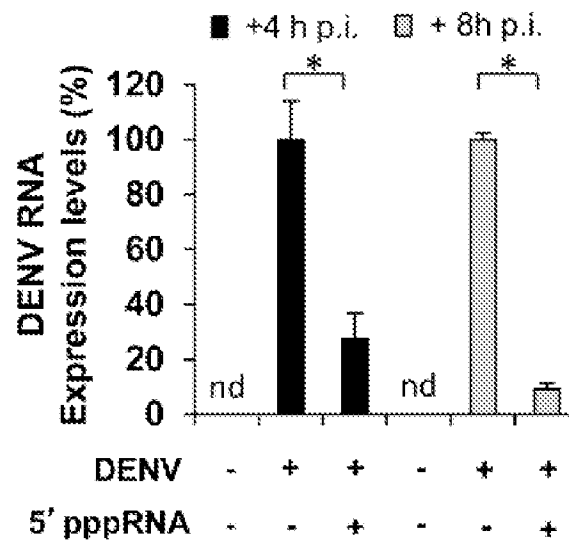


Figure 11C

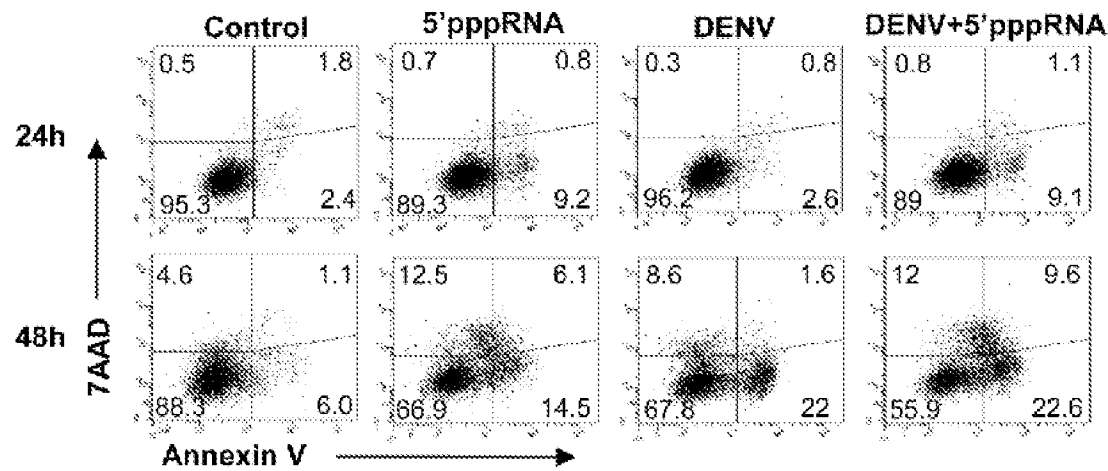


Figure 11D

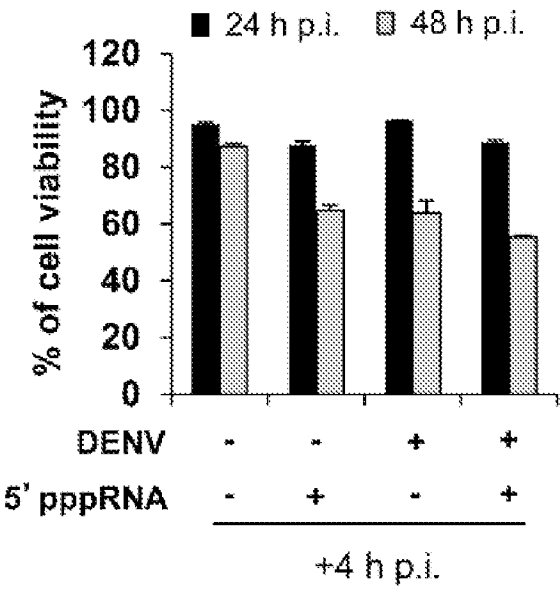


Figure 11E

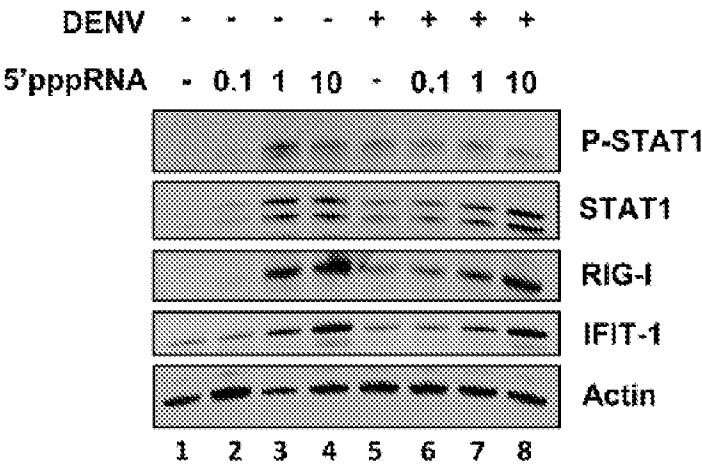


Figure 11F

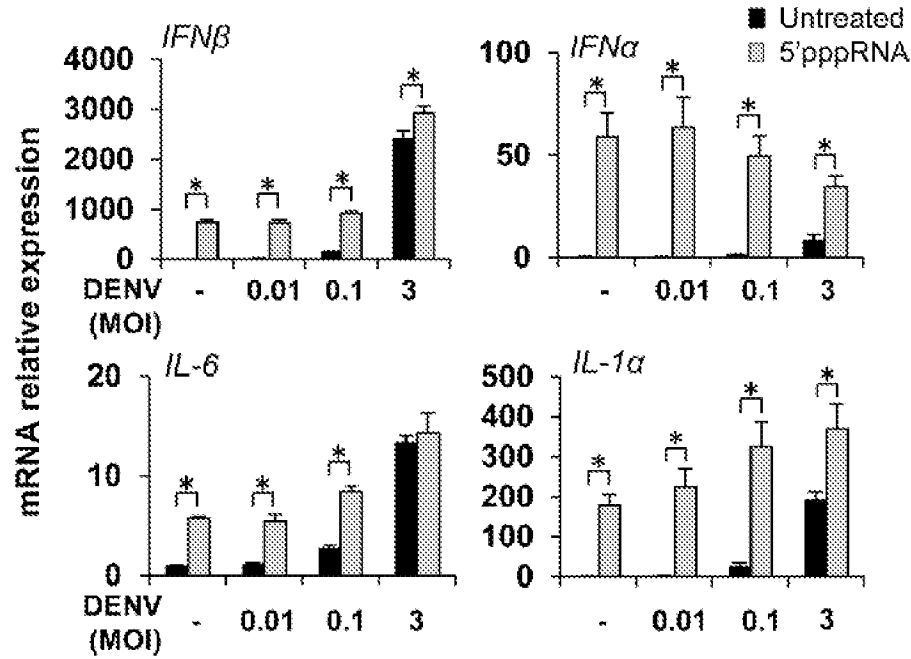


Figure 12A

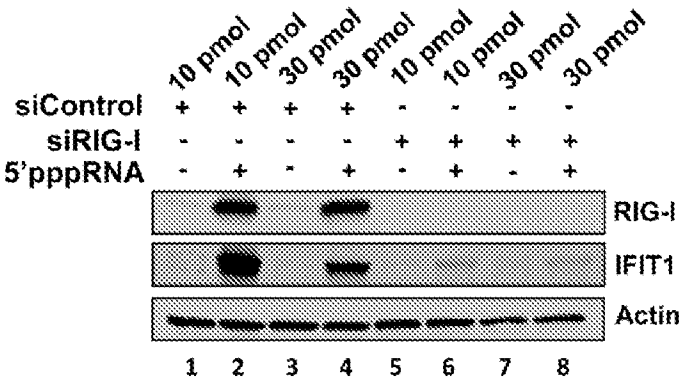


Figure 12B

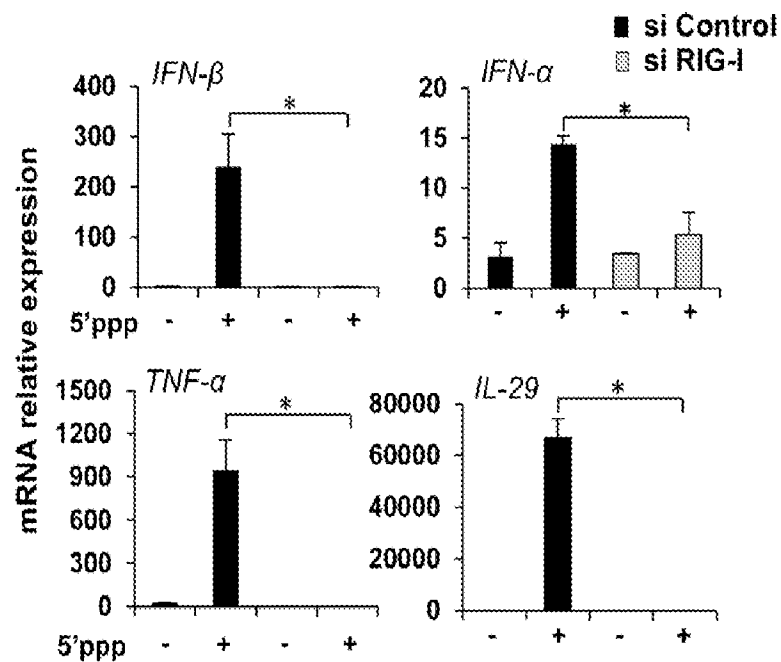


Figure 12C

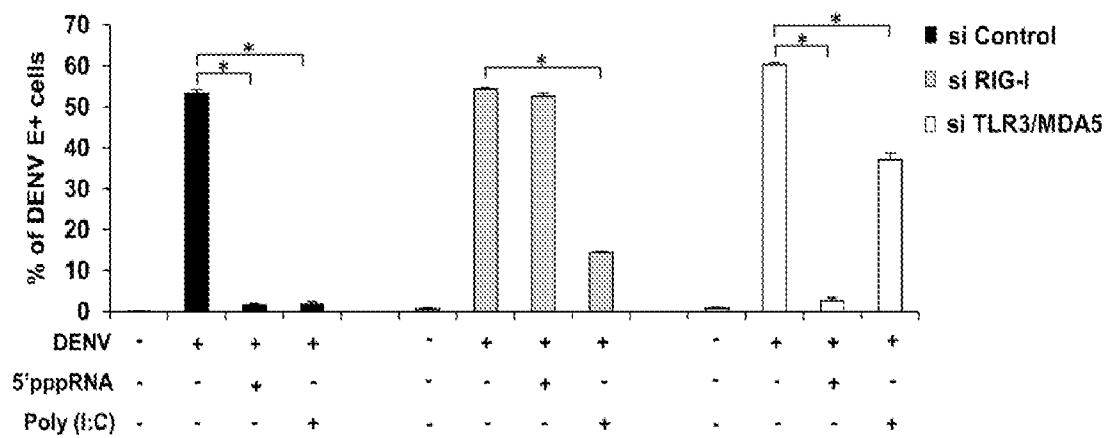


Figure 12D

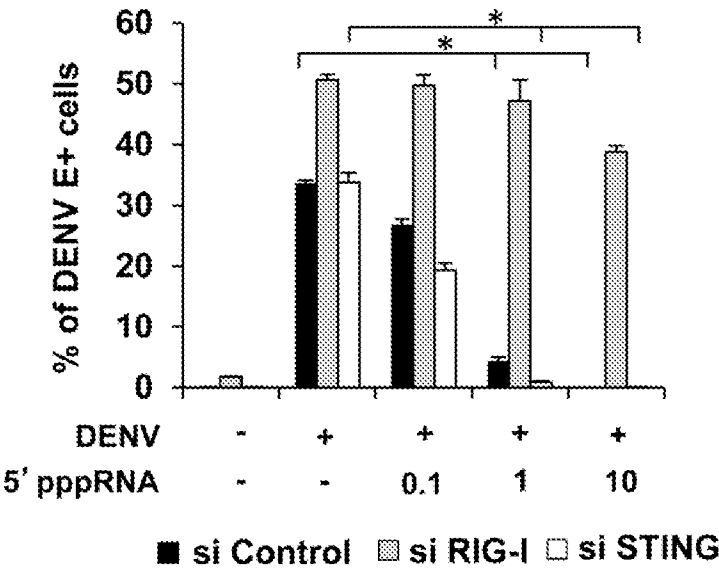


Figure 12E

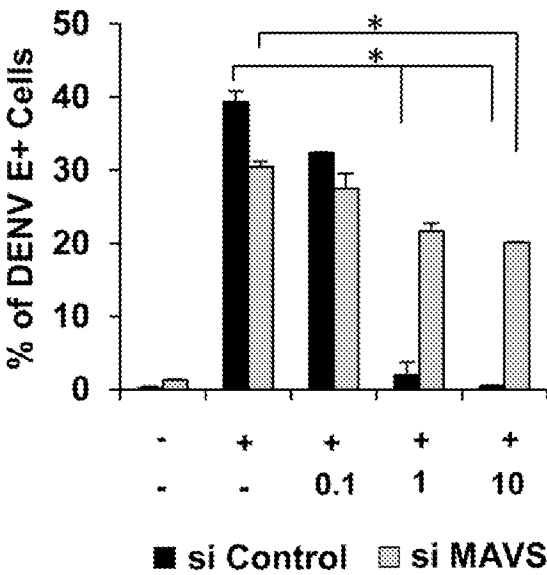


Figure 12F

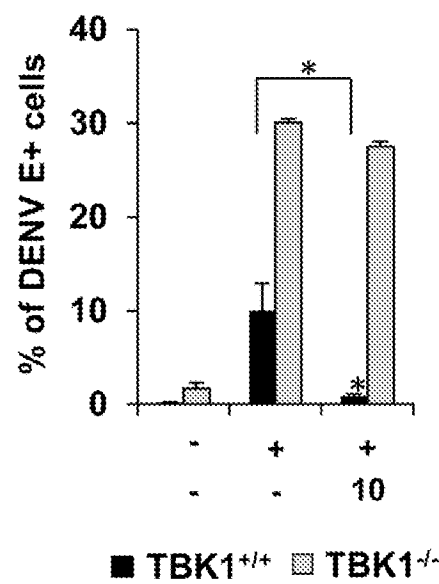


Figure 13A

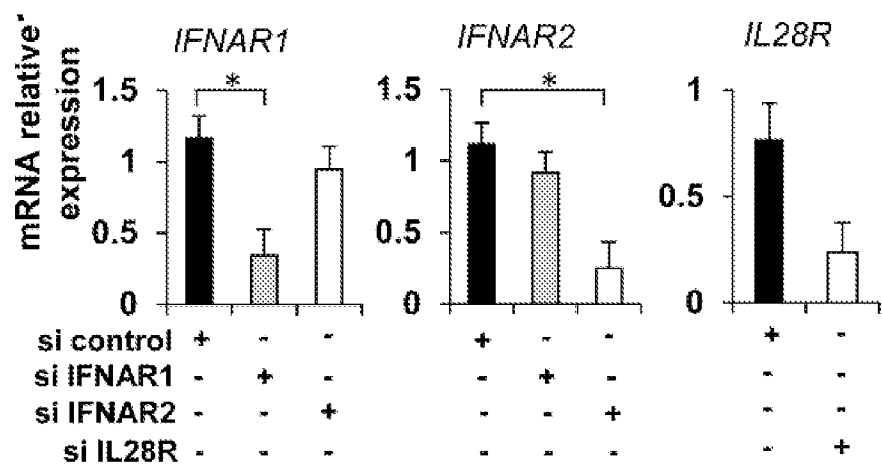


Figure 13B

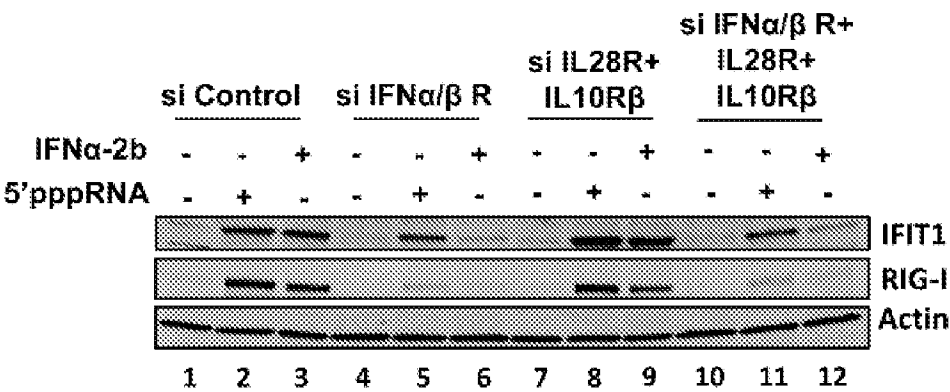


Figure 13C

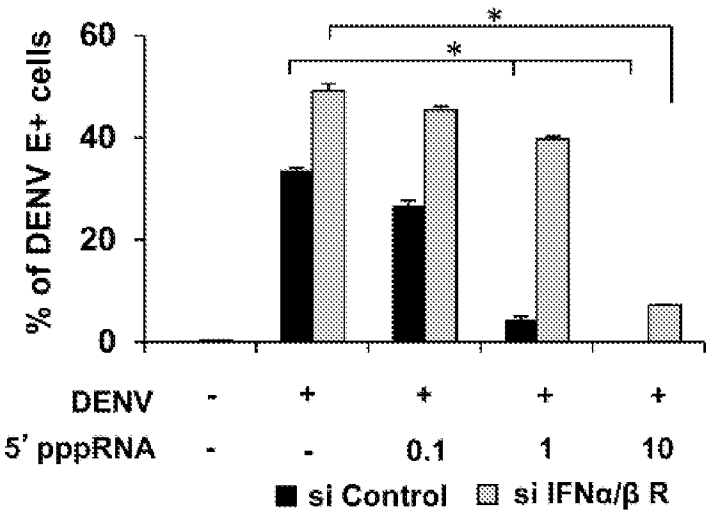


Figure 13D

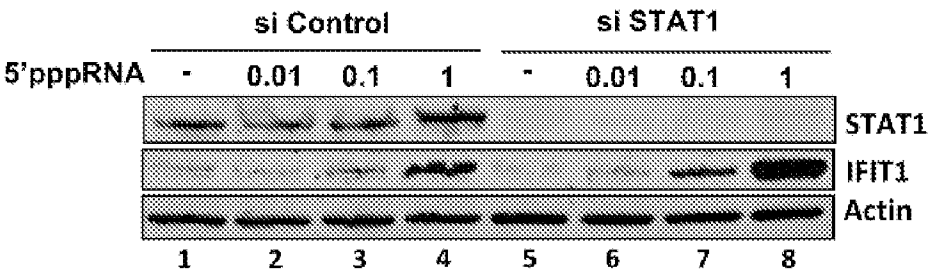


Figure 13E

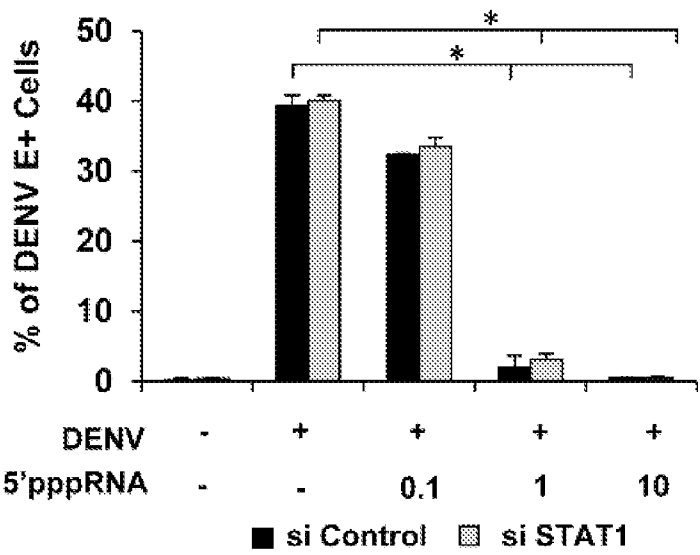


Figure 13F

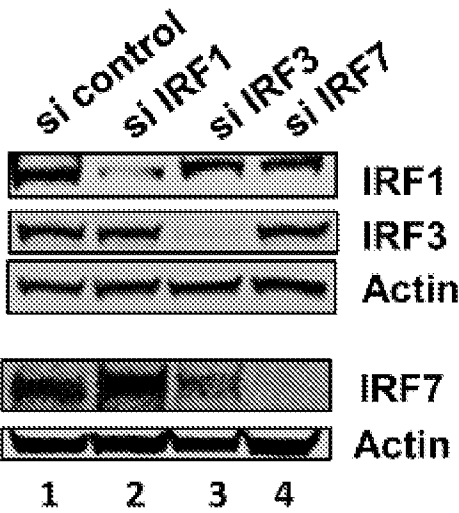


Figure 13G

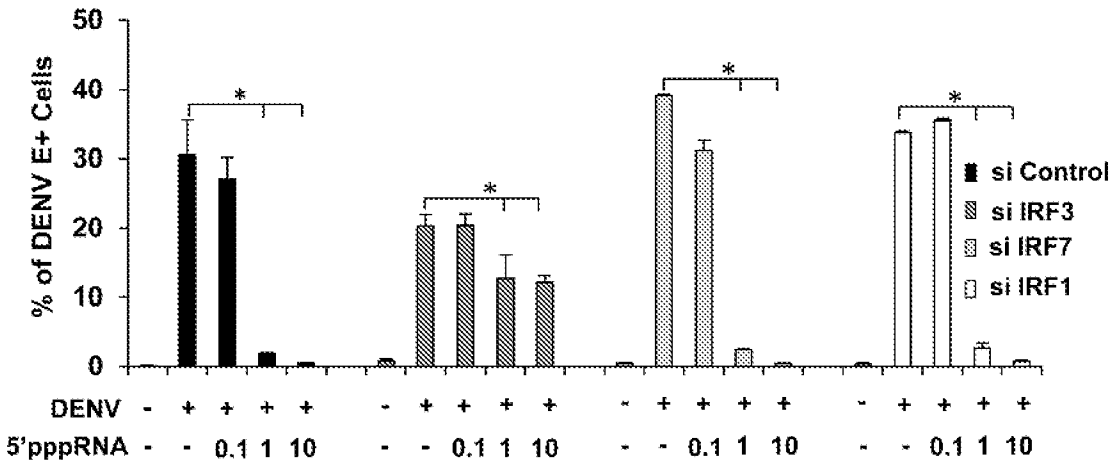


Figure 14A

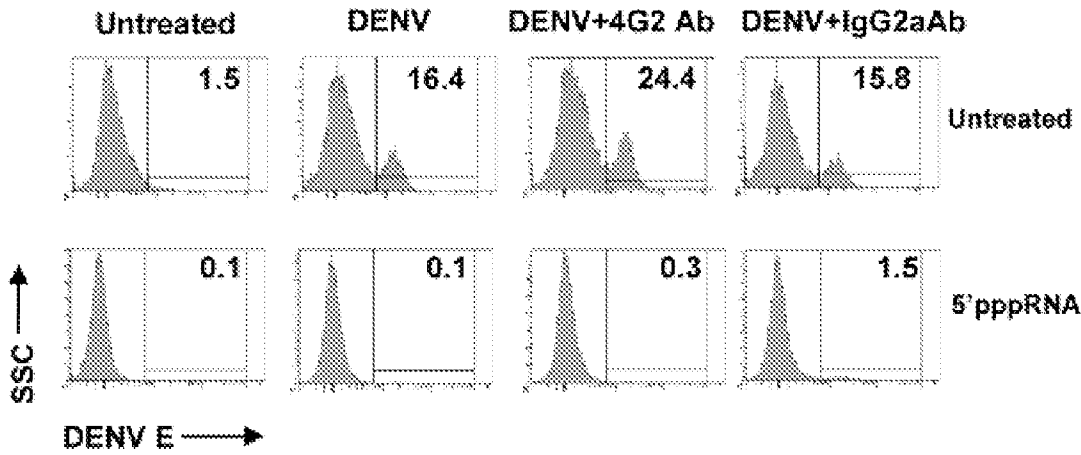


Figure 14B

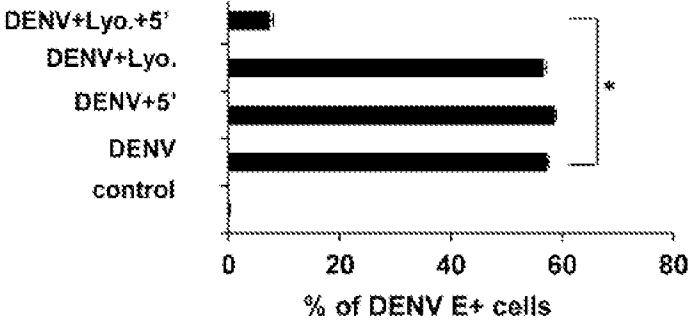


Figure 14C

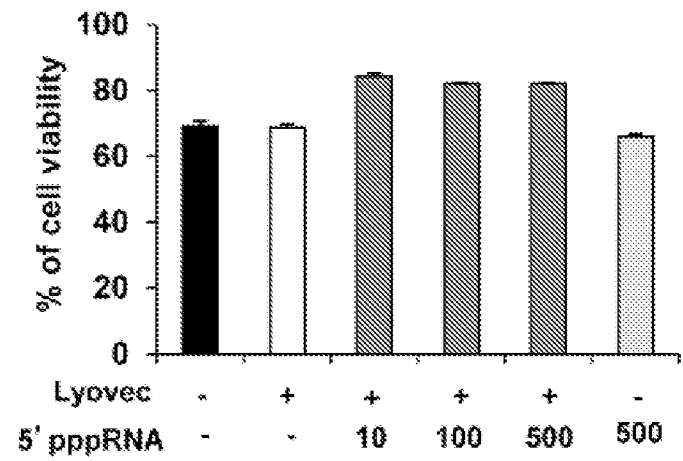


Figure 14D

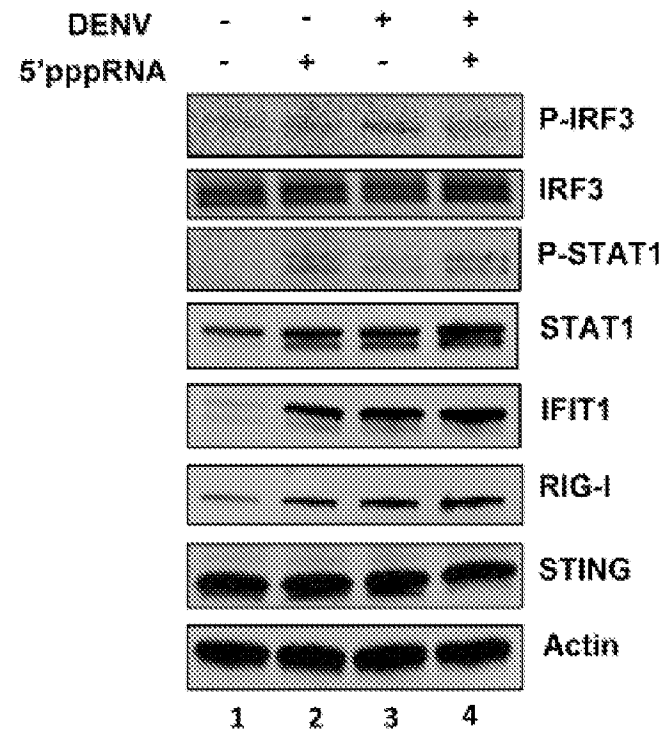


Figure 15A

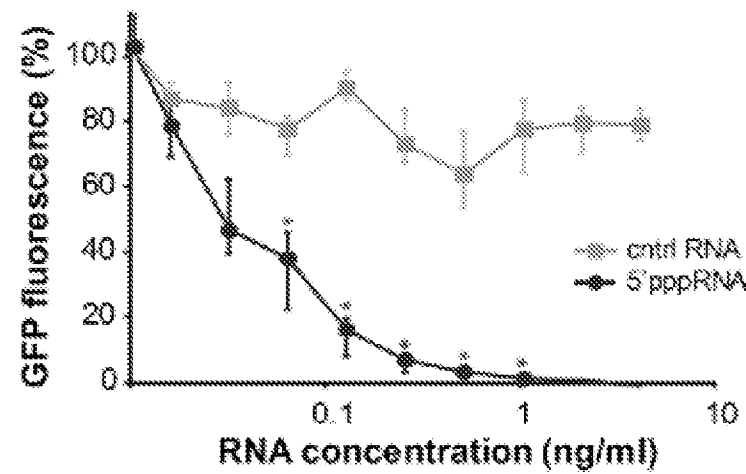


Figure 15B

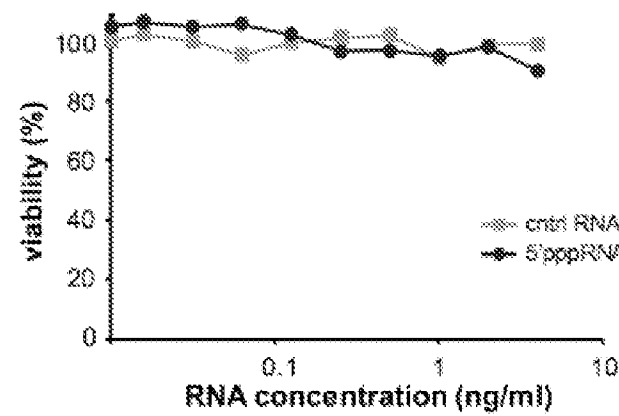


Figure 15C

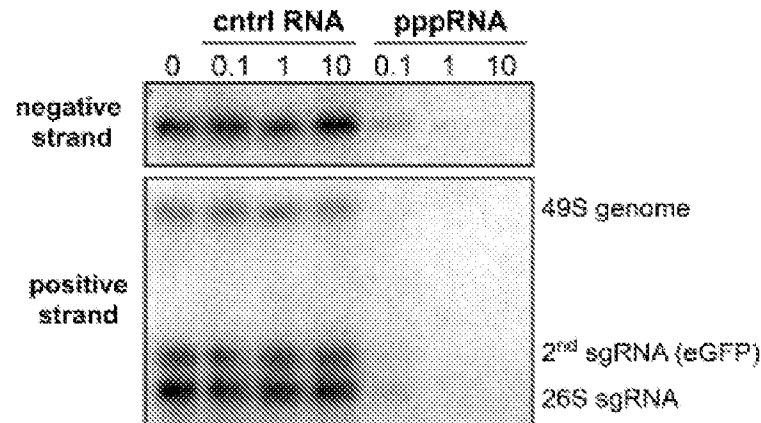


Figure 15D

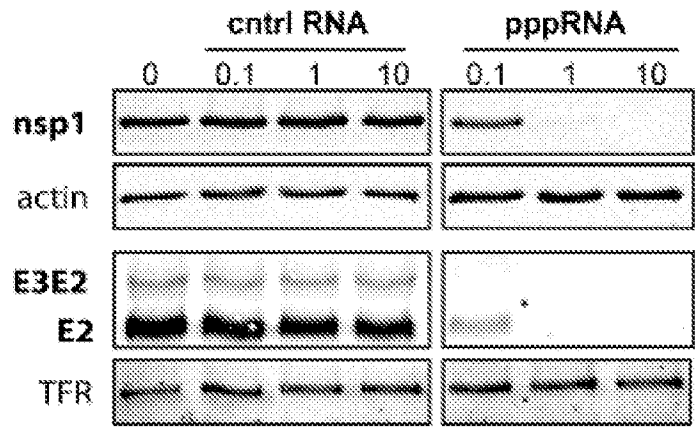


Figure 15E

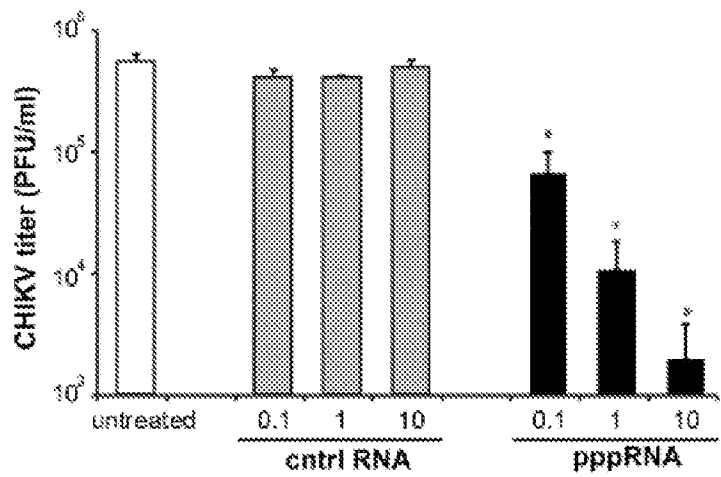


Figure 15F

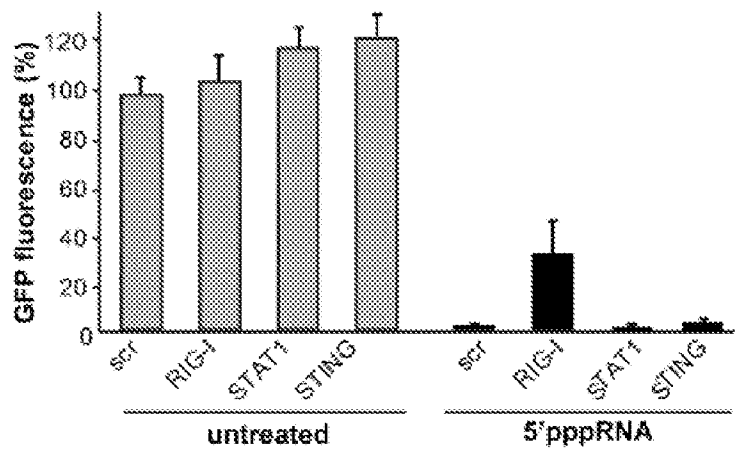


Figure 15G

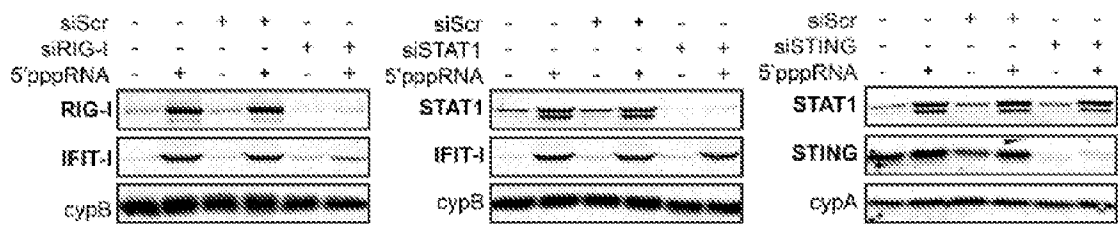


Figure 16A

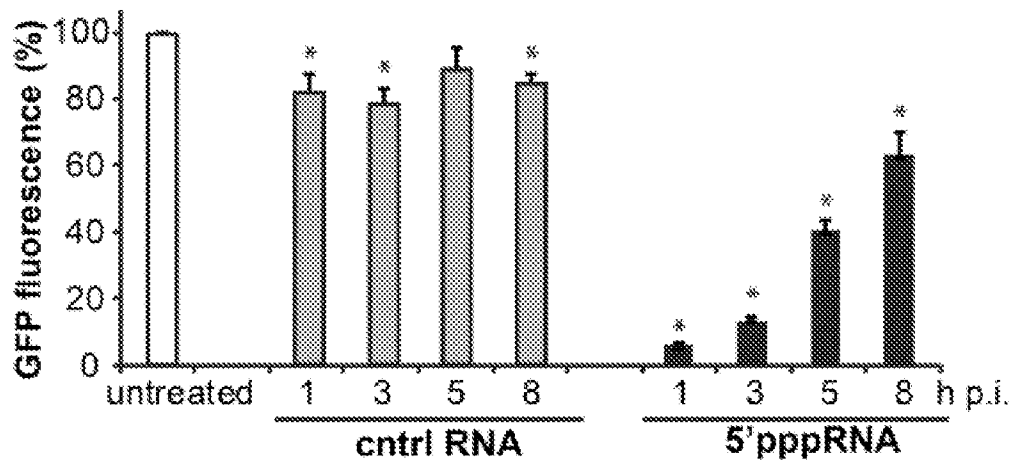


Figure 16B

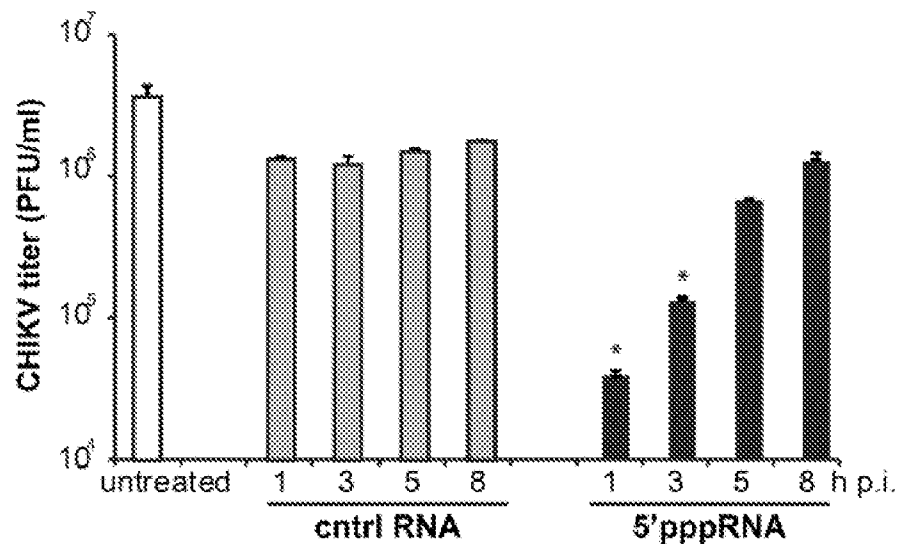
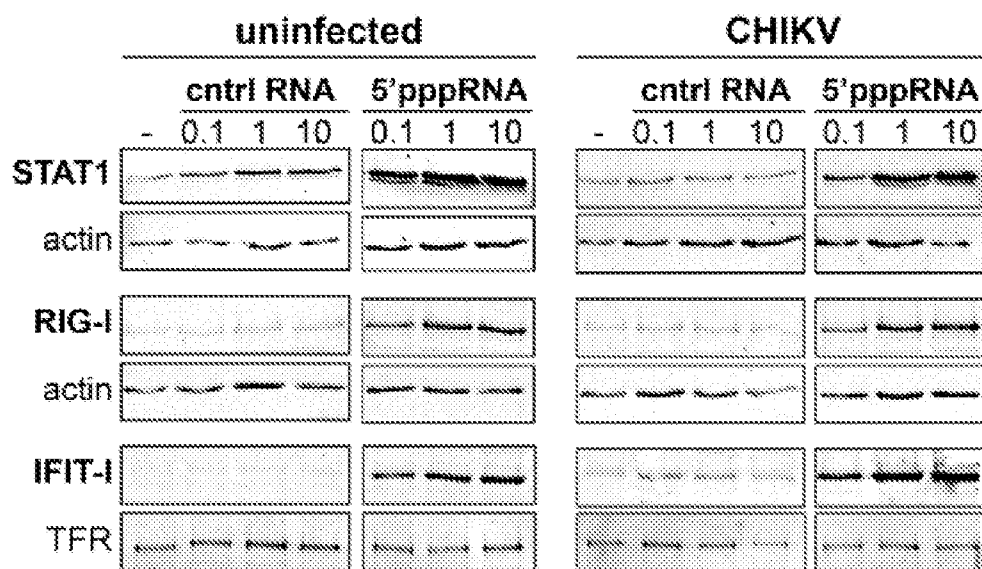


Figure 16C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/015774**A. CLASSIFICATION OF SUBJECT MATTER****C07H 21/02(2006.01)i, C12N 15/11(2006.01)i, A61K 31/7105(2006.01)i, A61K 48/00(2006.01)i**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07H 21/02; A61K 31/7105; A61K 39/00; A61K 31/713; A61K 38/21; C12N 15/11; A61K 48/00

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

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

eKOMPASS(KIPO internal) & Keywords:5'-triphosphate, oligoribonucleotide, viral infection, RIG-I signaling, VSV virus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2011-0165123 A1 (HARTMANN, GUNTHER et al.) 7 July 2011 See abstract; claims 1, 8, 10-11, 21-22; paragraphs [0041]-[0043], and [0342].	1-19
A	SCHLEE, MARTIN et al., "Recognition of 5' Triphosphate by RIG-I Helicase Requires Short Blunt Double-Stranded RNA as Contained in Panhandle of Negative-Strand Virus", 17 July 2009, Immunity, Vol. 31, Issue 1, Pages 25-34 See pages 25, 32.	1-19
A	US 2012-0288476 A1 (HARTMANN, GUNTHER et al.) 15 November 2012 See abstract; claim 1; paragraph [0575].	1-19
A	US 2012-0107272 A1 (MANOHARAN, MUTHIAH et al.) 3 May 2012 See abstract; claim 59.	1-19
A	POECK, HENDRIK et al., "5' -triphosphate-siRNA: turning gene silencing and Rig-I activation against melanoma", NATURE MEDICINE, 2 November 2008, Vol. 14, No. 11, Pages 1256-1263 See pages 1256, 1262.	1-19
A	NCBI, GenBank accession no. FJ478454.1 (28 December 2012) See whole document.	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

27 May 2014 (27.05.2014)

Date of mailing of the international search report

27 May 2014 (27.05.2014)

Name and mailing address of the ISA/KR

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Telephone No. +82-42-481-8150



INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/015774**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 21 is unclear since it refers to multiple dependent claim which is not searchable due to not being drafted in accordance with the third sentence of Rule 6.4(a).
3. ☒ Claims Nos.: 20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/015774

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0165123 A1	07/07/2011	EP 2123757 A1	25/11/2009
		EP 2123757 B1	31/10/2012
		EP 2297323 A1	23/03/2011
		JP 2011-522526 A	04/08/2011
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US 2012-0288476 A1	15/11/2012	AU 2007-283022 A1	14/02/2008
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		CA 2660232 A1	14/02/2008
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		EP 1920775 B1	19/12/2012
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		EP 2344514 A2	20/07/2011
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		US 2012-0220761 A1	30/08/2012
		WO 2010-028079 A2	11/03/2010
		WO 2010-028079 A3	17/06/2010
		WO 2011-028218 A1	10/03/2011