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**DICKEY et al.**(10) **Pub. No.: US 2022/0096627 A1**(43) **Pub. Date: Mar. 31, 2022**(54) **COMPOSITIONS AND METHODS FOR  
TREATING VIRAL INFECTIONS THROUGH  
STIMULATED INNATE IMMUNITY IN  
COMBINATION WITH ANTIVIRAL  
COMPOUNDS**(60) Provisional application No. 62/053,013, filed on Sep.  
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Apr. 25, 2019, now abandoned, which is a continu-  
ation of application No. 14/860,205, filed on Sep. 21,  
2015, now Pat. No. 10,286,065.(57) **ABSTRACT**Embodiments are directed to compositions and methods for  
treating viral infections.**Specification includes a Sequence Listing.**

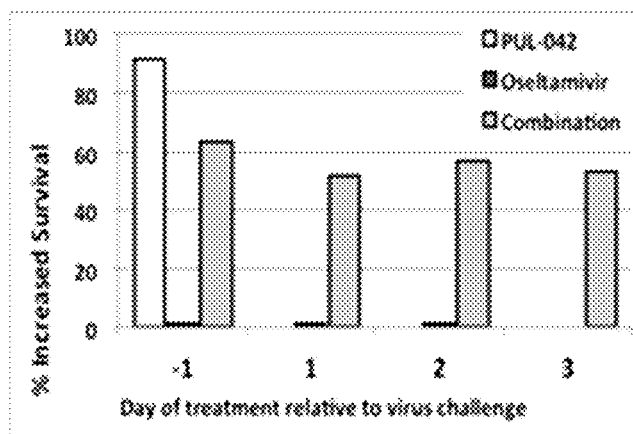


FIG. 1

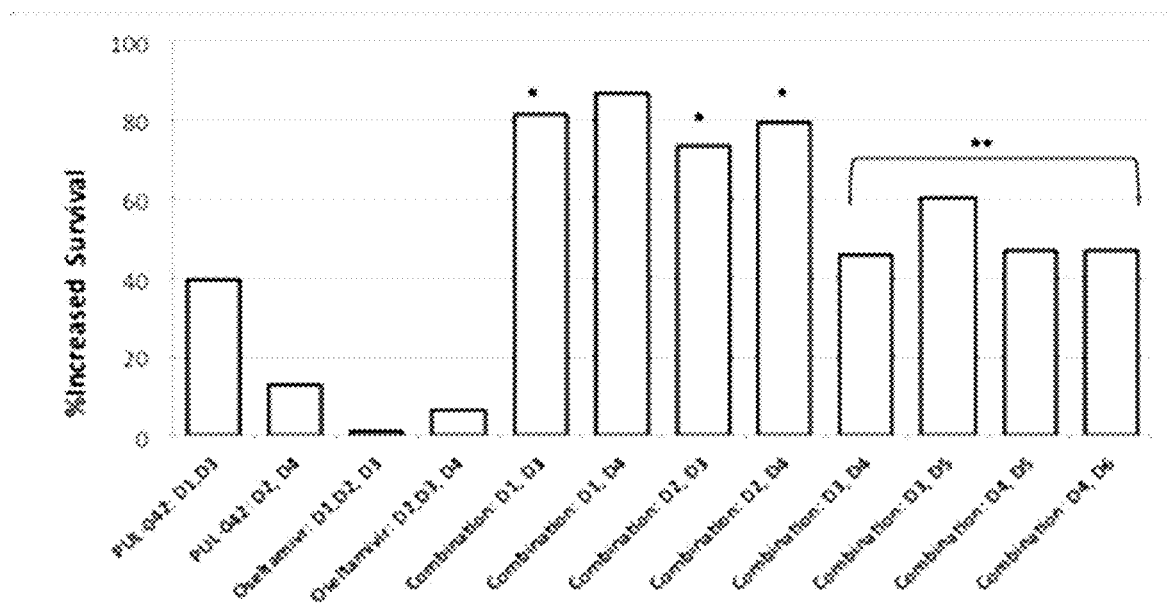


FIG. 2

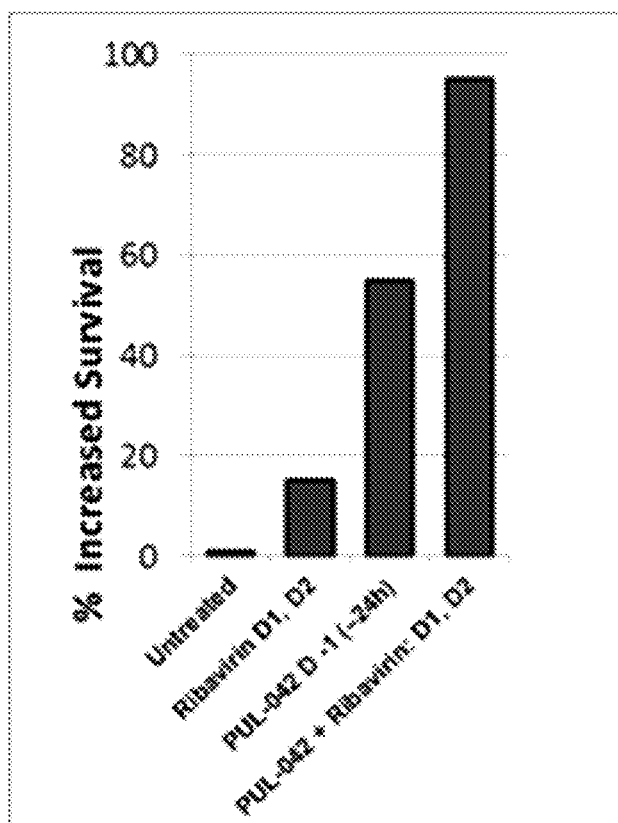


FIG. 3

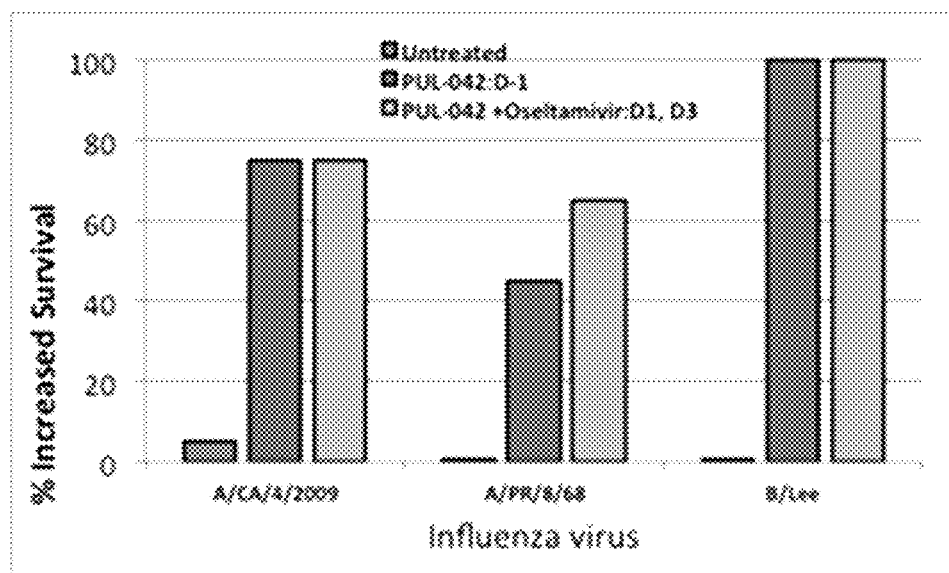


FIG. 4

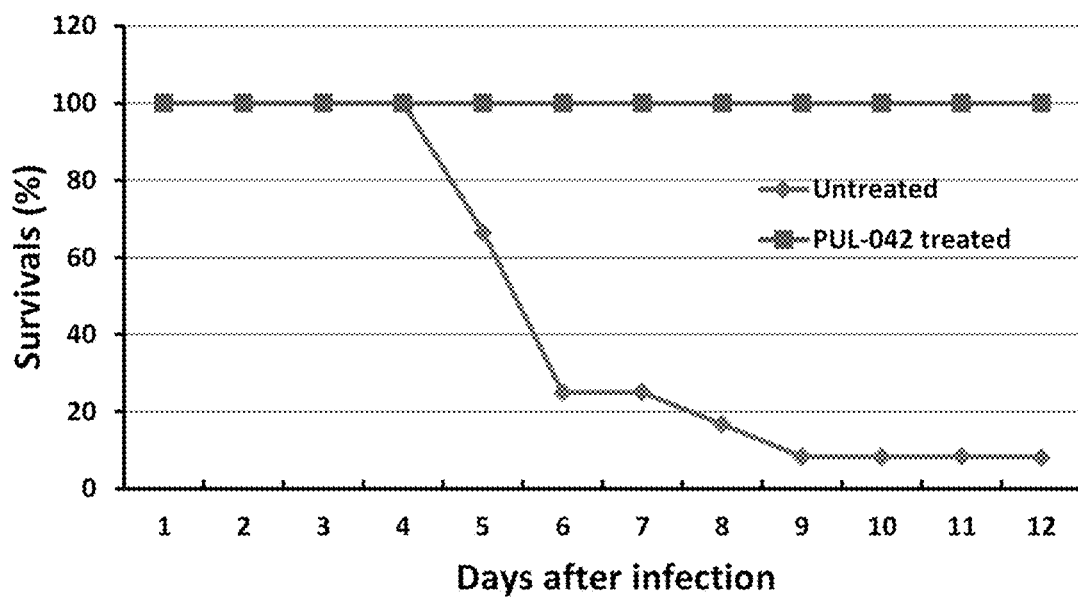


FIG. 5

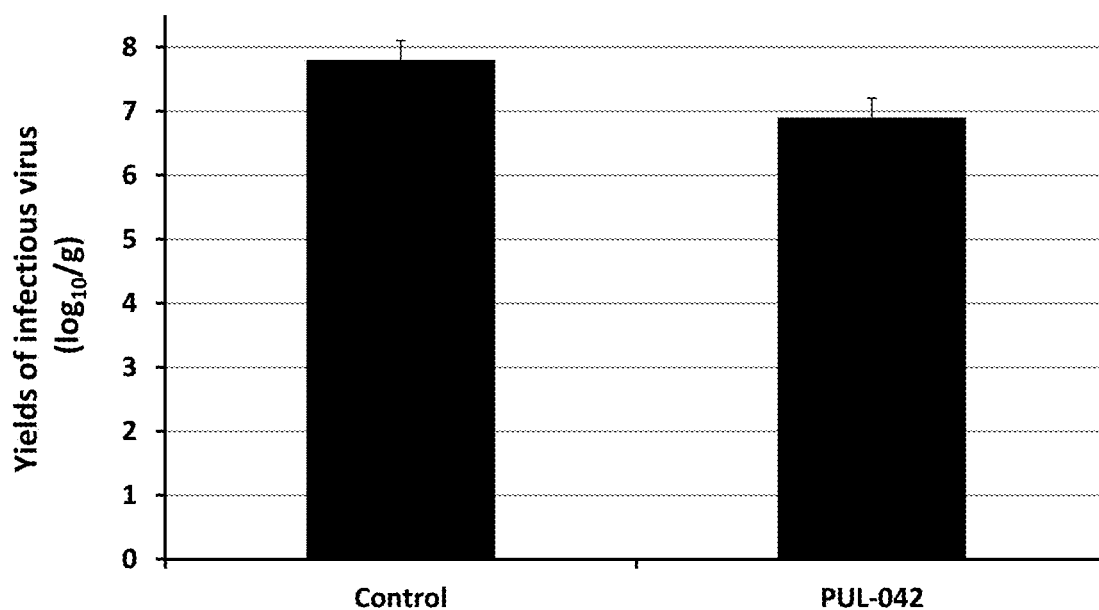


FIG. 6

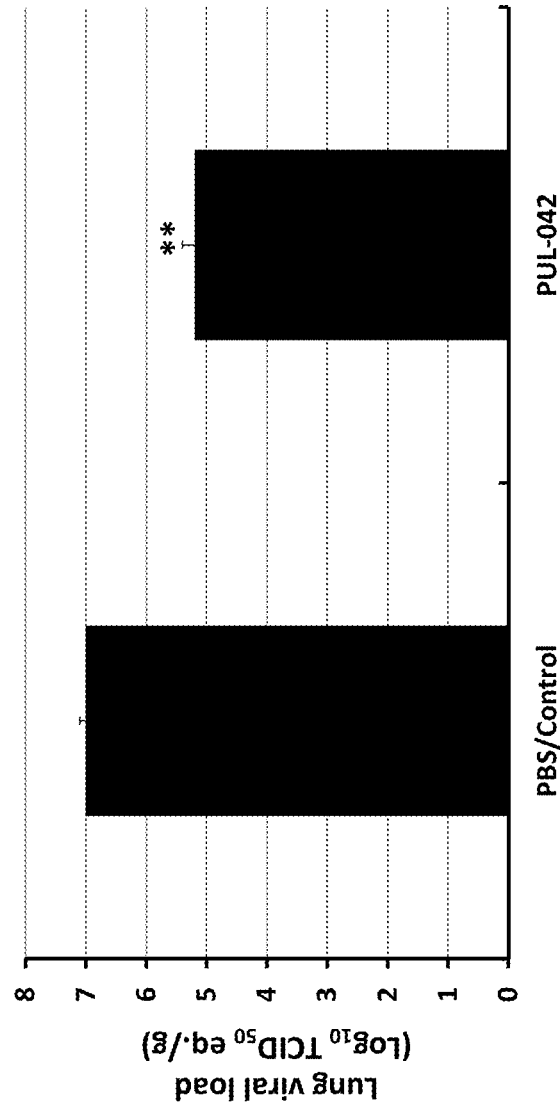


FIG. 7

**COMPOSITIONS AND METHODS FOR  
TREATING VIRAL INFECTIONS THROUGH  
STIMULATED INNATE IMMUNITY IN  
COMBINATION WITH ANTIVIRAL  
COMPOUNDS**

PRIORITY

**[0001]** This application is a continuation from U.S. application Ser. No. 16/394,097, filed Apr. 25, 2019, which is a continuation from U.S. application Ser. No. 14/860,205 filed Sep. 21, 2015, now U.S. Pat. No. 10,286,065, which claims priority to U.S. Provisional Applications 62/053,013 filed Sep. 19, 2014 and 62/053,610 filed Sep. 22, 2014, each of which are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY  
FUNDED RESEARCH

**[0002]** This invention was made with government support under grant number 1R43HL118926-01A1, 1DP2HL123229-01, and 1R01HL117976-01A1 awarded by the National Heart Lung and Blood Institute or the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

I. Field of the Invention

**[0003]** The present invention relates generally to the fields of virology, immunology, and antimicrobial pharmacotherapy. More particularly the compositions and methods of the invention related to increasing the resistance of an individual to viral infection.

II. Background

**[0004]** The susceptibility of the lungs to infection arises from the architectural requirements of gas exchange. To support ventilation, humans continuously expose about 80 m<sup>2</sup> lung surface area to the external environment. Lungs are exposed not only to air, but also the particles, droplets, and pathogens that are suspended in the air. Unlike cutaneous surfaces that are wrapped in impermeable skin or the gastrointestinal tract with a thick adsorbent blanket of mucus, the lungs present a large environmental interface with a minimal barrier defense. A more substantial barrier is precluded by the demand for unimpeded gaseous diffusion.

**[0005]** Despite their structural vulnerability, the lungs generally defend themselves successfully against infection through a variety of mechanical, humoral, and cellular mechanisms (Knowles et al., 2002; Martin and Frevert, 2005; Rogan, et al., 2006; Travis, et al., 2001); (Mizgerd, 2008; Bals and Hiemstra, 2004; Bartlett et al., 2008; Hiemstra, 2007; Hippenstiel et al., 2006; Schutte and McCray, 2002). Most inhaled microbial pathogens fail to penetrate to the alveoli due to impaction against the airway walls, where they are entrapped by mucus and then expelled via the mucociliary escalator system (Knowles et al., 2002). For those pathogens that escape this fate, the constitutive presence of antimicrobial peptides in the airway lining fluid limits their growth (Rogan, et al., 2006; Travis, et al., 2001). Alveolar macrophages that reside in the most distal airspaces are able to ingest these organisms, thereby clearing the lungs from a potential infection.

**[0006]** Though often regarded as passive gas exchange barriers, the airway and alveolar epithelia supplement the baseline lung defenses by undergoing remarkable local structural and functional changes when pathogenic stimuli are encountered. In response to viral, fungal, or allergic inflammation, airway secretory cells rapidly increase their height and fill their apical cytoplasm with secretory granules, a process termed inflammatory metaplasia (Evans et al., 2004; Williams et al., 2006). In the presence of pathogens, the alveolar epithelia activate their plasmalemmal systems and secretory machinery, thereby engaging leukocytes in lung protection (Evans et al., 2005). Perhaps most importantly, microbial interactions with respiratory epithelial pattern recognition receptors causes numerous microbicidal products to be expressed into the airway lining fluid, including defensins, cathelicidins, lysozyme, and reactive oxygen species (Rogan et al., 2006; Forteza et al., 2005; Akinbi et al., 2000; Bals 15 and Hiemstra, 2004; Bals and Hiemstra, 2006). It is of note that pneumonia (bacterial or viral) is the leading cause of death from infection worldwide.

**[0007]** There is a need for additional methods and compositions for inhibiting and/or treating viral infections.

SUMMARY

**[0008]** Certain embodiments are directed to compositions and methods for treating viral infections. In certain aspects the viral infection is a viral infection of the lungs. Other embodiments are directed to delivery devices containing an anti-viral composition(s). In certain aspects the delivery devices contain a formulation with activity against a broad spectrum of viruses. In a further aspect a delivery device can contain a formulation comprising one or more anti-viral drugs that target a specific family of viruses. Studies have shown that the combination treatments described herein are mechanism independent and that administration of a lipopeptide(s) and immune stimulatory oligonucleotide(s) can be co-administered or combined with a variety of antivirals having a variety of therapeutic mechanisms and targets. Thus, lipopeptide/oligonucleotide compositions and treatments can be effectively combined with wide variety of antivirals and are not limited to any particular antiviral.

**[0009]** Certain embodiments are directed to compositions that increase resistance of a subject to viruses when administered to the subject. Additional embodiments are directed to methods of using such compositions to attenuate viral infection in the subject. Thus, embodiments include, but are not limited to compositions, formulations, and methods for the enhancement of a mammalian (e.g., a human) subject's biological defenses against viral infection. In certain aspects compositions are administered or deposited in an effective amount in the lungs of a subject. In certain aspects the compositions and methods provide a rapid and temporal increase in resistance to infection and/or augmentation of biological defenses against viral infection. Attenuation of viral infection can be by inhibiting, treating, or preventing virus infection or replication or survival. In specific embodiments the subject is a human patient.

**[0010]** Aspects described here increase resistance to infection and enhance the defenses of the lung and respiratory tract of a subject. A subject administered a composition described herein is afforded a therapeutic, prophylactic, or therapeutic and prophylactic response to a potentially infecting virus.

**[0011]** Certain embodiments are directed to formulations or co-formulations of active components to provide for an anti-viral effect. In certain aspects a co-formulation comprises one or more (a) lipopeptide(s), (b) immune stimulatory oligonucleotide(s), or (c) antiviral drug(s). In certain aspects the anti-viral compositions contain an effective amount of at least one, two, or three of the following: (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s). In certain aspects one or more lipopeptides can be included in a formulation. In a further aspect one or more stimulatory oligonucleotides can be included in a formulation. In still a further aspect one or more anti-viral drugs can be included in a formulation. The term stimulatory oligonucleotide and immune stimulatory oligonucleotide are used interchangeably to refer to an immune stimulatory oligonucleotide. In certain aspects the lipopeptide and stimulatory oligonucleotide are co-formulated or administered simultaneously, i.e. lipopeptide/stimulatory oligonucleotide co-administration. In a further aspect the lipopeptide/stimulatory oligonucleotide co-administration is administered in conjunction with administration of an additional antiviral drug or therapy. "Administered in conjunction" or "coadministration" as used herein refers to administration of two or more active agents in a manner that will allow them to be present together in-vivo for period of time. Accordingly, while the term "coadministration" includes simultaneous administration of two or more active agents, and administration from a single formulation, it is to be understood that it is not limited thereto.

**[0012]** In certain aspects a lipopeptide is selected from diacyl and triacyl lipopeptides. In certain aspects a lipopeptide is FSL-1; Pam3Cys (tripalmitoyl-S-glyceryl cysteine); S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine; (S)-[2,3-bis(palmitoyloxy)-(2-R,S)-propyl]-Npalmitoyl-(R)-Cys-(S)-Ser-(Lys)4-hydroxytrihydrochloride; Pam3Cys-Ser-Ser-Asn-Ala; Pam3Cys-Ser-(Lys)4; Pam3Cys-Ala-Gly; Pam3Cys-Ser-Gly; Pam3Cys-Ser; Pam3Cys-OMe; Pam3Cys-OH; PamCAG (palmitoyl-Cys ((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH); or Pam2CSK4 (Pam2CSK4, dipalmitoyl-S-glyceryl cysteine-serine-(lysine)4), Pam2Cys-Ser-(Lys)4). In certain aspects the lipopeptide is PAM2CSK4.

**[0013]** In certain aspects a stimulatory oligonucleotide is a type A, B, or C oligodeoxynucleotide (ODN). In certain aspects the stimulatory oligonucleotide is a type C ODN. In a further aspect the ODN is ODN2395 (tcgtcggttttcgcgcgccg (SEQ ID NO:1) or ODNM362 (tcgtcggttcgaacgacgttgat (SEQ ID NO:2) or ODN10101 (tcgtcggttttcgcgcgccg (SEQ ID NO:3).

**[0014]** In certain aspects an antiviral drug is a drug that effects the biology of a virus and attenuates or inhibits attachment, entry, replication, shedding, latency or a combination thereof. In a further aspect the antiviral drug can be a viral mimetic, a nucleotide analog, a sialidase inhibitor, or a protease inhibitor. In certain aspects the anti-viral drug is a neuraminidase inhibitor or nucleotide analog. In a particular aspect the anti-viral drug is amantadine, rimantadine, ribavirin, zanamivir, or oseltamivir. In certain aspects the antiviral drug is a small molecule, or an antibody or antibody fragment.

**[0015]** In certain embodiments the lipopeptide is PAM2CSK4; the stimulatory oligonucleotide is ODN2395 (tcgtcggttttcgcgcgccg (SEQ ID NO:1) or ODNM362 (tcgtcggttcgaacgacgttgat (SEQ ID NO:2) or ODN10101

(tcgtcggttttcgcgcgccg (SEQ ID NO:3); and the antiviral drug is amantadine, rimantadine, ribavirin, zanamivir, or oseltamivir.

**[0016]** In one embodiment, the anti-viral compositions contain about 0.1, 0.5, 1, 5, or 10% to about 1, 5, 10, or 20% by weight of at least one of (a) lipopeptide(s), (b) stimulatory oligonucleotide(s), or (c) antiviral drug(s).

**[0017]** In certain aspects a formulation can comprise a lipopeptide in an amount that is at least, less than or about 0.1, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 55% by weight or volume (or any range derivable therein).

**[0018]** In certain aspects a formulation can comprise a stimulatory oligonucleotide in an amount that is at least, less than or about 0.1, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 55% by weight or volume (or any range derivable therein).

**[0019]** In certain aspects a formulation can comprise an anti-viral drug in an amount that is at least, less than or about 0.1, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 55% by weight or volume (or any range derivable therein).

**[0020]** In one embodiment, the antiviral compositions contain at least one of (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s). In another embodiment, the anti-viral compositions contain at least two (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s). In still another embodiment the anti-viral compositions contain a (a) lipopeptide, (b) stimulatory oligonucleotide, and (c) antiviral drug(s).

**[0021]** In one embodiment, the weight ratio of (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s) relative to each other in the anti-viral compositions includes or is at least or at most 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 parts lipopeptide (or any range derivable therein) to, to at least, or to at most 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 parts stimulatory oligonucleotide (or any range derivable therein) to, to at least or to at most 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 parts anti-viral drug (or any range derivable therein). In certain aspects a formulation can comprise about 4 parts lipopeptide, about 1 part stimulatory oligonucleotide. In additional embodiments, there is also about or at least about, or at most about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 parts antiviral drug (or any range derivable therein).

**[0022]** In certain embodiments a composition can comprise, comprise at least or comprise at most 0, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10 g of lipopeptide (or any range derivable therein) per 1.5, or 10 mL; 0, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10 g of stimulatory oligonucleotide (or any range derivable therein) per 1, 5, or 10 mL; and/or 0, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 10, 50 up to 100 g of antiviral drug(s) (or any range derivable therein) per 1, 5, or 10 mL.

**[0023]** Certain embodiments are directed to methods of treating, inhibiting, or attenuating a viral infection in a subject who has or is at risk for developing such an infection. The methods comprising administering an effective amount of an anti-viral composition described herein.

**[0024]** In certain embodiments a lipopeptide and stimulatory oligonucleotide can be administered via the respiratory system and an anti-viral drug can be administered orally or intravascularly.

**[0025]** Certain embodiments are directed to compositions capable of being administered to the respiratory tract 1, 2, 3, 4, or more times a day, week, or month (or any combination derivable therein).

**[0026]** In other aspects a composition is administered in a nebulized formulation. The (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s) can be administered in an amount, selected independently for each component, from about, about at least or about at most 0.1, 1, 5, 10, 50  $\mu\text{g}$  or  $\text{mg/kg}$  to about, about at least or about at most 5, 10, 50, 100  $\mu\text{g}$  or  $\text{mg/kg}$  of the subject's body weight, including all values and ranges there between.

**[0027]** Compositions described herein can be administered via the respiratory tract. Methods of the invention include the administration of a composition by inhalation or other methods of administration to the upper and/or lower respiratory tract. In certain aspects, the anti-viral composition is administered in a nebulized or aerosolized formulation. In a further aspect the composition is aerosolized or nebulized or in a form that can be inhaled by or instilled in a subject. The composition can be administered by inhalation or inspiration. The anti-viral composition, including (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s), can be administered in an amount of from about, or at least or at most about, 0.01, 0.05, 0.1, 25 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70  $\mu\text{g}$  or  $\text{mg/kg}$  (or any range derivable therein) to about, or at least or at most about, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 200  $\mu\text{g}$  or  $\text{mg/kg}$  (or any range derivable therein) of the subject's body weight. In other aspects, a subject can be administered about, or at least or at most about 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 200  $\mu\text{g}$  or  $\text{mg}$  (or any range derivable therein) of (a) lipopeptide, (b) stimulatory oligonucleotide, or (c) antiviral drug(s) individually or in combination (total amount). The subject can be at risk of exposure to or exposed to a virus. Still further embodiments include methods where the composition is administered before; after; during; before and after; before and during; during and after; before, after, and during exposure or suspected exposure or heightened risk of exposure to the virus. The subject can be exposed to a bioweapon or to an opportunistic pathogen. In particular aspects the subject is immunocompromised, such as an infant, a cancer patient, or an AIDS patient. In certain aspects the subject is located in an area having or at risk of having a viral outbreak.

**[0028]** Certain embodiments include a pharmaceutical composition comprising or consisting essentially of PAM2CSK4, ODNM362, and optionally an antiviral agent, that is formulated for aerosolized or nebulized delivery. In certain embodiments the antiviral agent is ribavirin or oseltamivir. Methods include treating a patient for a virus infection comprising administering to the patient effective amounts of PAM2CSK4 and ODNM362, and optionally administering an antiviral agent, wherein the PAM2CSK4 and ODNM362 are administered to the patient as an aerosol or with a nebulizer. In certain embodiments, treatment of the virus infection does not include an active agent other than a lipopeptide, a stimulatory oligonucleotide (such as a Class C ODN, including ODNM362), and an antiviral drug. In certain aspects the compositions or methods specifically exclude an antigen or immunogen targeting a specific virus or group viruses.

**[0029]** In certain aspects the virus is an Adenoviridae, Coronaviridae, Filoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Paramyxovirinae, Pneumovirinae, Picornaviridae, Poxviridae, Retroviridae, or Togaviridae virus. In a further aspect a virus is Parainfluenza,

Influenza (seasonal, swine, avian, etc.), Marburg, Ebola, Severe acute respiratory syndrome coronavirus, Yellow fever virus, Human respiratory syncytial virus, Hantavirus, measles, MERS, rhinovirus, human metapneumovirus, or Vaccinia virus. In other aspects the virus is influenza, RSV, or parainfluenza virus. In a further aspect the virus can be a Severe acute respiratory syndrome coronavirus (SARS-COV) or Middle Eastern Respiratory Syndrome coronavirus (MERS-COV).

**[0030]** The terms "attenuating," "inhibiting," "reducing," or "prevention," or any variation of these terms, when used in the claims and/or the specification includes any measurable decrease or complete inhibition to achieve a desired result, e.g., reduction in post-exposure viral survival, load, or growth.

**[0031]** As used herein, "an effective amount" means the concentration or quantity or level of the active compound(s) of the present invention that can attain a particular medical end, such as control or destruction of virally-infected cells or viruses, without producing unacceptable toxic symptoms. The term "effective amount" also refers to the quantity of an active compound(s) that is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. The specific "effective amount" can vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed.

**[0032]** Other embodiments of the invention are discussed throughout this application. Any embodiment discussed with respect to one aspect of the invention applies to other aspects of the invention as well and vice versa. Each embodiment described herein is understood to be embodiments of the invention that are applicable to all aspects of the invention. It is contemplated that any embodiment discussed herein can be implemented with respect to any method or composition of the invention, and vice versa. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

**[0033]** The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

**[0034]** Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

**[0035]** The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

**[0036]** As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0037] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### DESCRIPTION OF THE DRAWINGS

[0038] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of the specification embodiments presented herein.

[0039] FIG. 1. Effect of PUL-042 on percent survival compared to untreated control mice following lethal challenge of influenza infection. The x-axis indicates the day on which PUL-042 and/or oseltamivir was administered relative to influenza infection.

[0040] FIG. 2. Effect of timing of multiple doses of combination treatment with PUL-042 and oseltamivir on survival of a lethal influenza challenge. The x-axis indicates the day of initiation of treatment and timing of subsequent treatments relative to the influenza challenge. \* Statistically significant difference from untreated controls. \*\* Combined results of treatments starting at D3 or D4, Ave.=49.8%,  $P<0.001$ . \*\*\* Combination D1 and D4, while a strong result, was performed only once and statistical significance was not determined.

[0041] FIG. 3. Effect of PUL-042 combination treatment with ribavirin on survival of a lethal influenza challenge. Initiating treatment on D1 after infection increased the percent survival to 95% compared to untreated controls.

[0042] FIG. 4. Effect of PUL-042 on percent survival following lethal challenge of three influenza viruses. Fifteen outbred NIH Swiss-Webster mice were used in each group. The x-axis indicates the day on which PUL-042 and/or oseltamivir was administered relative to infection challenge.

[0043] FIG. 5. Prior aerosolized PUL-042 treatment fully protects mice against lethal SARS-COV infection (~24 hrs; challenge dose:  $5 \times LD_{50}$ ).

[0044] FIG. 6. Prior aerosolized PUL-042 treatment reduces pulmonary yields of infectious SARS-COV (~24 hrs; challenge dose:  $5 \times LD_{50}$ ; 3 dpi).

[0045] FIG. 7. Prior treatment with aerosolized PUL-042 significantly reduced the viral loads in MERS-COV-challenged mice (3 dpi).

#### DESCRIPTION

[0046] The immune system is the system of specialized cells and organs that protect an organism from outside biological influences. When the immune system is functioning properly, it protects the body against microbial infections, and destroys cancer cells and foreign substances. If the immune system weakens, its ability to defend the body also weakens, allowing pathogens to grow in the body.

[0047] The immune system is often divided into: (a) an innate immunity comprised of components that provide an immediate "first-line" of defense to continuously ward off pathogens and (b) an adaptive (acquired) immunity com-

prising the production of antibodies and production or stimulation of T-cells specifically designed to target particular pathogens. Using adaptive immunity the body can develop over time a specific immunity to particular pathogen(s). This response takes days to develop, and so is not effective at preventing an initial invasion, but it will normally prevent any subsequent infection, and also aids in clearing up longer-lasting infections.

[0048] In response to certain inflammatory stimuli, the secretory cells of the airway epithelium of mice and humans rapidly undergo a remarkable change in structure termed inflammatory metaplasia. Most of the structural changes can be ascribed to increased production of secreted, gel-forming mucins, while additional macromolecules with functions in mucin secretion, microbial killing or inflammatory signaling are also upregulated. The physiologic function of this response is thought to be augmentation of local defenses against microbial and helminthic pathogens, although that hypothesis has received only limited formal testing. Paradoxically, excessive production and secretion of gel-forming mucins is a major cause of airflow obstruction in common inflammatory diseases of the airways such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD). The stimulation of innate immunity without the production or with the reduced production of mucin provides an additional method of attenuating infection of the respiratory tract by preventing and/or treating a viral infection of a subject.

#### III. Anti-Viral Compositions and Treatments

[0049] The compositions and methods of the present invention may be used in the context of a number of therapeutic or prophylactic applications. In order to increase the effectiveness of a treatment with the compositions described or to augment the protection of another therapy (second therapy), e.g., vaccination or antimicrobial therapy, it may be desirable to combine these compositions and methods with other agents and methods effective in the treatment, reduction of risk of infection, or prevention of diseases and pathologic conditions, for example, anti-viral treatments. In certain aspects a plurality of components are formulated in a composition for administration to a subject in need of such.

[0050] Administration of a composition described to a subject will follow general protocols for the administration via the respiratory system, and the general protocols for the administration of a particular secondary therapy will also be followed, taking into account the toxicity, if any, of the treatment. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as vaccination, may be applied in combination with the described therapies.

[0051] In certain embodiments a composition can comprise one or more of (a) lipopeptide, (b) stimulatory oligonucleotide, and/or (c) anti-viral drug(s), in various combinations. Combination being in the form of co-formulation or alternatively co-administration of components.

[0052] A. Lipopeptides

[0053] Lipopeptides include synthetic triacylated and diacylated lipopeptides. The peptide component can include single amino acids such as cysteine or short 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid peptides. In certain aspects the peptide can include one or more amino terminal serine and 1, 2, 3, 4, or more carboxy terminal asparagine, glycine, alanine,

lysine residues or combinations thereof. A nonlimiting example of lipopeptide is FSL-1 (a synthetic lipoprotein derived from *Mycoplasma salivarium* 1). Pam3Cys (tripalmitoyl-S-glyceryl cysteine)S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteine, where “Pam3” is “tripalmitoyl-Sglyceryl”) (Aliprantis et al., 1999), derivatives of Pam3Cys (S-[2,3-bis(palmitoyloxy)-(2-R,S)-propyl]-Npalmitoyl-(R)-Cys-(S)-Ser-(Lys)4-hydroxytrihydrochloride; Pam3Cys-Ser-Ser-Asn-Ala; Pam3Cys-Ser-(Lys)4; Pam3Cys-Ala-Gly; Pam3Cys-Ser-Gly; Pam3Cys-Ser; Pam3Cys-OMe; Pam3Cys-OH; PamCAG, palmitoyl-Cys ((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH; Pam2CSK4 (Pam2CSK4, dipalmitoyl-S-glyceryl cysteine-serine-(lysine)4), Pam2Cys-Ser-(Lys)4; and the like. Synthetic lipopeptides have been described in the literature. See, e.g., Kellner et al. (1992); Seifer et al. (1990); Lee et al. (2003).

#### [0054] B. Stimulatory Oligonucleotide

[0055] Stimulatory oligonucleotides include nucleic acids comprising the sequence 5'-CG-3' (a “CpG nucleic acid”), in certain aspects C is unmethylated. The terms “polynucleotide,” and “nucleic acid,” as used interchangeably herein in the context of stimulatory oligonucleotides molecules, refer to a polynucleotide of any length, and encompasses, inter alia, single- and double-stranded oligonucleotides (including deoxyribonucleotides, ribonucleotides, or both), modified oligonucleotides, and oligonucleosides, alone or as part of a larger nucleic acid construct, or as part of a conjugate with a non-nucleic acid molecule such as a polypeptide. Thus a stimulatory oligonucleotide may be, for example, single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA).

[0056] A stimulatory oligonucleotide may comprise at least one nucleoside comprising an L-sugar. The L-sugar may be deoxyribose, ribose, pentose, deoxypentose, hexose, deoxyhexose, glucose, galactose, arabinose, xylose, lyxose, or a sugar “analog” cyclopentyl group. The L-sugar may be in pyranosyl or furanosyl form.

[0057] Stimulatory oligonucleotides generally do not provide for, nor is there any requirement that they provide for, expression of any amino acid sequence encoded by the polynucleotide, and thus the sequence of a stimulatory oligonucleotide may be, and generally is, non-coding. Stimulatory oligonucleotide may comprise a linear double or single-stranded molecule, a circular molecule, or can comprise both linear and circular segments. Stimulatory oligonucleotide may be single-stranded, or may be completely or partially double-stranded.

[0058] In some embodiments, a stimulatory oligonucleotide for use in a subject method is an oligonucleotide, e.g., consists of a sequence of from about 5 nucleotides to about 200 nucleotides, from about 10 nucleotides to about 100 nucleotides, from about 12 nucleotides to about 50 nucleotides, from about 15 nucleotides to about 25 nucleotides, from 20 nucleotides 15 to about 30 nucleotides, from about 5 nucleotides to about 15 nucleotides, from about 5 nucleotides to about 10 nucleotides, or from about 5 nucleotides to about 7 nucleotides in length. In some embodiments, a stimulatory oligonucleotide that is less than about 15 nucleotides, less than about 12 nucleotides, less than about 10 nucleotides, or less than about 8 nucleotides in length is associated with a larger molecule.

[0059] In general, a stimulatory oligonucleotide used in a subject composition comprises at least one unmethylated CpG motif. The relative position of any CpG sequence in a polynucleotide in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position).

[0060] In some embodiments, a stimulatory oligonucleotide comprises a central palindromic core sequence comprising at least one CpG sequence, where the central palindromic core sequence contains a phosphodiester backbone, and where the central palindromic core sequence is flanked on one or both sides by phosphorothioate backbone-containing polyguanosine sequences.

[0061] In other embodiments, a stimulatory oligonucleotide comprises one or more TCG sequences at or near the 5' end of the nucleic acid; and at least two additional CG dinucleotides. In some of these embodiments, the at least two additional CG dinucleotides are spaced three nucleotides, two nucleotides, or one nucleotide apart. In some of these embodiments, the at least two additional CG dinucleotides are contiguous with one another. In some of these embodiments, the stimulatory oligonucleotide comprises (TCG)<sub>n</sub>, where n=1 to 3, at the 5' end of the nucleic acid. In other embodiments, the stimulatory oligonucleotide comprises (TCG)<sub>n</sub>, where n=1 to 3, and where the (TCG)<sub>n</sub> sequence is flanked by one nucleotide, two nucleotides, three nucleotides, four nucleotides, or five nucleotides, on the 5' end of the (TCG)<sub>n</sub> sequence.

[0062] Exemplary consensus CpG motifs useful in the invention include, but are not necessarily limited to: 5'-Purine-Purine-(C)-(G)-Pyrimidine-Pyrimidine-3', in which the stimulatory oligonucleotide comprises a CpG motif flanked by at least two purine nucleotides (e.g., GG, GA, AG, AA, II, etc.) and at least two pyrimidine nucleotides (CC, TT, CT, TC, UU, etc.); 5'-Purine-TCG-Pyrimidine-Pyrimidine-3'; 5'-TCG-N-N-3'; where N is any base; 5'-Nx(CG)<sub>n</sub>Ny, where N is any base, where x and y are independently any integer from 0 to 200, e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-15, 16-20, 21-25, 25-30, 30-50, 50-75, 75-100, 100-150, or 150-200; and n is any integer that is 1 or greater, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or greater. 5'-Nx(TCG)<sub>n</sub>Ny, where N is any base, where x and y are independently any integer from 0 to 200, e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11-15, 16-20, 21-25, 25-30, 30-50, 50-75, 75-100, 100-150, or 150-200; and n is any integer that is 1 or greater, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or greater. 5'-(TCG)<sub>n</sub>-3', where n is any integer that is 1 or greater, e.g., to provide a TCG-based TLR9 ligand (e.g., where n=3, the polynucleotide comprises the sequence 5'TCGNNTCGNNTCG-3'; SEQ ID NO:4); 5 Nm-(TCG)<sub>n</sub>-Np-3', where N is any nucleotide, where m is zero, one, two, or three, where n is any integer that is 1 or greater, and where p is one, two, three, or four; 5 Nm-(TCG)<sub>n</sub>-Np-3', where N is any nucleotide, where m is zero to 5, and where n is any integer that is 1 or greater, where p is four or greater, and where the sequence N-N-N-N comprises at least two CG dinucleotides that are either contiguous with each other or are separated by one nucleotide, two nucleotides, or three nucleotides; and 5'Purine-Purine-CG-Pyrimidine-TCG-3'.

[0063] Where a stimulatory oligonucleotide comprises a sequence of the formula: 5'-Nm(TCG)<sub>n</sub>-Np-3', where N is any nucleotide, where m is zero to 5, and where n is any integer that is 1 or greater, where p is four or greater, and where the sequence N—N—N—N comprises at least two CG dinucleotides that are either contiguous with each other or

are separated by one nucleotide, two nucleotides, or three nucleotides, exemplary stimulatory oligonucleotide useful in the invention include, but are not necessarily limited to: (1) a sequence of the formula in which  $n=2$ , and Np is NNCGNNCG; (2) a sequence of the formula in which  $n=2$ , and Np is AACGTTCG; (3) a sequence of the formula in which  $n=2$ , and Np is TTCGAACG; (4) a sequence of the formula in which  $n=2$ , and Np is TACGTACG; (5) a sequence of the formula in which  $n=2$ , and Np is A TCGA TCG; (6) a sequence of the formula in which  $n=2$ , and Np is CGCGCGCG; (7) a sequence of the formula in which  $n=2$ , and Np is GCCGGCCG; (8) a sequence of the formula in which  $n=2$ , and Np is CCCGGGCG; (9) a sequence of the formula in which  $n=2$ , and Np is GGCGCCCG; (10) a sequence of the formula in which  $n=2$ , and Np is CCCGTTTCG; (11) a sequence of the formula in which  $n=2$ , and Np is GGCGTTTCG; (12) a sequence of the formula in which  $n=2$ , and Np is TTCGCCCCG; (13) a sequence of the formula in which  $n=2$ , and Np is TTCGGGCG; (14) a sequence of the formula in which  $n=2$ , and Np is AACGCCCCG; (15) a sequence of the formula in which  $n=2$ , and Np is AACGGGCG; (16) a sequence of the formula in which  $n=2$ , and Np is CCCGAACG; and (17) a sequence of the formula in which  $n=2$ , and Np is GGCGAACG; and where, in any of 1-17,  $m=$ zero, one, two, or three.

**[0064]** Where a nucleic acid TLR9 ligand comprises a sequence of the formula: 5'Nm(TCG)n-Np-3', where N is any nucleotide, where  $m$  is zero, one, two, or three, where  $n$  is any integer that is 1 or greater, and where  $p$  is one, two, three, or four, exemplary TLR9 ligands useful in the invention include, but are not necessarily limited to: (1) a sequence of the formula where  $m=$ zero,  $n=1$ , and Np is T-T-T; (2) a sequence of the formula where  $m=$ zero,  $n=1$ , and Np is T-T-T-T; (3) a sequence of the formula where  $m=$ zero,  $n=1$ , and Np is C-C-CC; (4) a sequence of the formula where  $m=$ zero,  $n=1$ , and Np is A-A-A-A; (5) a sequence of the formula where  $m=$ zero,  $n=1$ , and Np is A-G-A-T; (6) a sequence of the formula where Nm is T,  $n=1$ , and Np is T-T-T; (7) a sequence of the formula where Nm is A,  $n=1$ , and Np is T-T-T; (8) a sequence of the formula where Nm is C,  $n=1$ , and Np is T-T-T; (9) a sequence of the formula where Nm is G,  $n=1$ , and Np is T-T-T; (10) a sequence of the formula where Nm is T,  $n=1$ , and Np is A-T-T; (11) a sequence of the formula where Nm is A,  $n=1$ , and Np is 15 A-T-T; and (12) a sequence of the formula where Nm is C,  $n=1$ , and Np is A-T-T.

**[0065]** Stimulatory oligonucleotides useful in the invention include, but are not necessarily limited to, polynucleotides comprising one or more of the following nucleotide sequences:

AGCGCT, AGCGCC, AGCGTT, AGCGTC, AACGCT, AACGCC,  
AACGTT, AACGTC, GGCGCT, GGCGCC, GGCGTT, GGCGTC,  
GACGCT, GACGCC, GACGTT, GACGTC, GTCGCT, GTCGTC,  
GTCGTT, GTCGCC, ATCGTC, ATCGCT, ATCGTT, ATCGCC,  
TCGTCG, or TCGTCGTCG.

**[0066]** Additional stimulatory oligonucleotides useful in the invention include, but are not necessarily limited to, polynucleotides comprising one or more of the following nucleotide sequences:

TCGXXXX, TCGAXXX, XTCGXXX, XTCGAXX, TCGTCGA,  
TCGACGT, TCGAACG, TCGAGAT, TCGACTC, TCGAGCG,  
TCGATTT, TCGCTTT, TCGGTTT, TCGTTTT, TCGTCGT,  
ATCGATT, TTCGTTT, TTCGATT, ACGTTCG, AACGTTC,  
TGACGTT, TGTCGTT, TCGXXX, TCGAXX, TCGTCG, AACGTT,  
ATCGAT, GTCGTT, GACGTT, TCGXX, TCGAX, TCGAT,  
TCGTT, TCGTC, TCGA, TCGT, TCGX, and TCG  
(where "X" is any nucleotide).

**[0067]** Stimulatory oligonucleotides useful in the invention include, but are not necessarily limited to, polynucleotides comprising the following octameric nucleotide sequences:

AGCGCTCG, AGCGCCCG, AGCGTTCG, AGCGTCCG, AACGCTCG,  
AACGCCCCG, AACGTTTCG, AACGTCCG, GGCGCTCG, GGCGCCCCG,  
GGCGTTTCG, GGCGTCCG, GACGCTCG, GACGCCCG, GACGTTTCG,  
and GACGTCCG.

**[0068]** A stimulatory oligonucleotide useful in carrying out a subject method can comprise one or more of any of the above CpG motifs. For example, a stimulatory oligonucleotide useful in the invention can comprise a single instance or multiple instances (e.g., 2, 3, 4, 5 or more) of the same CpG motif. Alternatively, a stimulatory oligonucleotide can comprise multiple CpG motifs (e.g., 2, 3, 4, 5 or more) where at least two of the multiple CpG motifs have different consensus sequences, or where all CpG motifs in the stimulatory oligonucleotides have different consensus sequences.

**[0069]** A stimulatory oligonucleotide useful in the invention may or may not include palindromic regions. If present, a palindrome may extend only to a CpG motif, if present, in the core hexamer or octamer sequence, or may encompass more of the hexamer or octamer sequence as well as flanking nucleotide sequences.

**[0070]** In some embodiments, a stimulatory oligonucleotide is multimeric. A multimeric stimulatory oligonucleotide comprises two, three, four, five, six, seven, eight, nine, ten, or more individual (monomeric) stimulatory oligonucleotides, as described above, linked via noncovalent bonds, linked via covalent bonds, and either linked directly to one another, or linked via one or more spacers. Suitable spacers include nucleic acid and non-nucleic acid molecules, as long as they are biocompatible. In some embodiments, multimeric stimulatory oligonucleotide comprises a linear array of monomeric stimulatory oligonucleotides. In other embodiments, a multimeric stimulatory oligonucleotide is a branched, or dendrimeric, array of monomeric stimulatory oligonucleotides.

**[0071]** Stimulatory oligonucleotide modifications. A stimulatory oligonucleotide suitable for use in a subject composition can be modified in a variety of ways. For example, a stimulatory oligonucleotide can comprise backbone phosphate group modifications (e.g., methylphosphonate, phosphorothioate, phosphoramidate and phosphorodithioate internucleotide linkages), which modifications can, for example, enhance their stability in vivo, making them particularly useful in therapeutic applications. A particularly

useful phosphate group modification is the conversion to the phosphorothioate or phosphorodithioate forms of a stimulatory oligonucleotide. Phosphorothioates and phosphorodithioates are more resistant to degradation in vivo than their unmodified oligonucleotide counterparts, increasing the half-lives of the stimulatory oligonucleotides and making them more available to the subject being treated.

**[0072]** Other modified stimulatory oligonucleotides encompassed by the present invention include stimulatory oligonucleotides having modifications at the 5' end, the 3' end, or both the 5' and 3' ends. For example, the 5' and/or 3' end can be covalently or non-covalently associated with a molecule (either nucleic acid, non-nucleic acid, or both) to, for example, increase the bio-availability of the stimulatory oligonucleotide, increase the efficiency of uptake where desirable, facilitate delivery to cells of interest, and the like.

**[0073]** The terms “CpG-ODN,” “CpG nucleic acid,” “CpG polynucleotide,” and “CpG oligonucleotide,” used interchangeably herein, refer to a polynucleotide that comprises at least one 5'-CG-3' moiety, and in many embodiments comprises an unmethylated 5'-CG-3' moiety. In general, a CpG nucleic acid is a single- or double-stranded DNA or RNA polynucleotide having at least six nucleotide bases that may comprise, or consist of, a modified nucleotide or a sequence of modified nucleosides. In some embodiments, the 5'-CG-3' moiety of the CpG nucleic acid is part of a palindromic nucleotide sequence. In some embodiments, the 5'-CG-3' moiety of the CpG nucleic acid is part of a non-palindromic nucleotide sequence.

**[0074]** C. Anti-Viral Drugs.

**[0075]** In certain aspects an anti-viral drug(s) may be used in combination with or as a component of an anti-viral composition (co-formulated with other components) described herein. Anti-viral drugs are a class of medication used specifically for treating viral infections and they should be distinguished from viricides, which actively deactivate virus particles outside the body. Most of the antivirals now available are designed to help deal with HIV, herpes viruses, the hepatitis B and C viruses, and influenza A and B viruses. Anti-viral agents useful in embodiments include, but are not limited to, immunoglobulins, amantadine, interferons, nucleotide analogues, sialidase inhibitors and protease inhibitors. It is contemplated that one or more of these may be included in embodiments or they may be excluded from embodiments. In certain embodiments, the antiviral drug is one that inhibits the virus directly, instead of destroying or killing the virus. In other embodiments, an antiviral drug is not an immunoglobulin or agent that involves the immune system.

**[0076]** One anti-viral strategy is to interfere with the ability of a virus to infiltrate a target cell. This stage of viral replication can be inhibited by using agents that mimic the virus associated protein (VAP) and bind to the cellular receptors; or by using agents which mimic the cellular receptor and bind to the VAP. This includes anti-VAP antibodies, receptor anti-idiotypic antibodies, extraneous receptor and synthetic receptor mimics (viral mimetics). Two such “entryblockers” or “viral mimetics” are amantadine and rimantadine. In certain aspects amantadine, rimantadine, or compounds with similar mechanisms of action can be used in composition described herein. In a further aspect amantadine and rimantadine can be formulated as a treatment for influenza.

**[0077]** A second approach to anti-viral therapy is to target the processes that synthesize virus components after a virus invades a cell. One way of doing this is to develop nucleotide or nucleoside analogues that look like the building blocks of RNA or DNA, but deactivate the enzymes that synthesize the RNA or DNA once the analog is incorporated. Nucleotide analogs include, but are not limited to ribavirin, vidarabine, acyclovir, gancyclovir, zidovudine, didanosine, zalcitabine, stavudine, and lamivudine. A number of anti-proliferative compounds are known to inhibit both cancers and viruses, thus other anti-proliferative compounds can be used as an anti-viral therapy.

**[0078]** Another approach is to inhibit sialidases (also referred to as neuraminidases). Sialidases hydrolyse alpha-(2/3)-, alpha-(2/6)-, alpha-(2/8)-glycosidic linkages of terminal sialic residues in oligosaccharides, glycoproteins, glycolipids, colominic acid, and synthetic substrates. Sialidases act as pathogenic factors in virus infections. Thus, sialidase inhibitors can be used to attenuate the ability of a virus to infect a subject.

**[0079]** Some viruses have a protease that cuts viral protein chains apart so they can be assembled into their final configuration. HIV includes a protease, and so considerable research has been performed to find “protease inhibitors” to attack HIV at that phase of its life cycle. Protease inhibitors became available in the 1990s and have proven effective.

**[0080]** The final stage in the life cycle of a virus is the release of mature viruses from the host cell. Two drugs (neuraminidase inhibitors, also referred to as sialidase inhibitors) named zanamivir (RELENZA™) and oseltamivir (TAMIFLU™) that have been introduced to treat influenza prevent the release of viral particles by blocking a molecule named neuraminidase that is found on the surface of flu viruses, and also seems to be constant across a wide range of flu strains.

**[0081]** Anti-viral drugs include, but are not limited to abacavir; acemannan; acyclovir; acyclovir sodium; adefovir; alovudine; alvircept sudotox; amantadine hydrochloride; amprenavir; arantoin; arildone; atevirdine mesylate; avridine; cidofovir; cipamfylline; cytarabine hydrochloride; delavirdine mesylate; desciclovir; didanosine; disoxaril; edoxudine; efavirenz; envirodene; enviroxime; famciclovir; famotone hydrochloride; fiacitabine; fialuridine; fosarilate; trisodium phosphonoformate; fosfonet sodium; ganciclovir; ganciclovir sodium; idoxuridine; indinavir; kethoxal; lamivudine; lobucavir; memotone hydrochloride; methisazone; nelfinavir; nevirapine; penciclovir; pirodavid; ribavirin; rimantadine hydrochloride; ritonavir; saquinavir mesylate; somantadine hydrochloride; sorivudine; statolon; stavudine; tilorone hydrochloride; trifluridine; valacyclovir hydrochloride; vidarabine; vidarabine phosphate; vidarabine sodium phosphate; viroxime; zalcitabine; zidovudine; zidoviroxime, interferon, cyclovir, alpha-interferon, and/or beta globulin.

**[0082]** In certain embodiments the antiviral drug is ribavirin or high dose ribavirin. Ribavirin is an anti-viral drug that is active against a number of DNA and RNA viruses. It is a member of the nucleoside antimetabolite drugs that interfere with duplication of viral genetic material. Though not effective against all viruses, ribavirin has wide range of activity, including important activities against influenzas, flaviviruses, and agents of many viral hemorrhagic fevers.

**[0083]** Typically, the oral form of ribavirin is used in the treatment of hepatitis C, in combination with pegylated

interferon drugs. The aerosol form has been used in the past to treat respiratory syncytial virus-related diseases in children. However, its efficacy has been called into question by multiple studies, and most institutions no longer use it.

**[0084]** D. Other Agents

**[0085]** In certain aspects of the invention an anti-inflammatory agent may be used in combination with a composition described herein.

**[0086]** Steroidal anti-inflammatories for use herein include, but are not limited to fluticasone, beclomethasone, any pharmaceutically acceptable derivative thereof, and any combination thereof. As used herein, a pharmaceutically acceptable derivative includes any salt, ester, enol ether, enol ester, acid, base, solvate or hydrate thereof. Such derivatives may be prepared by those of skill in the art using known methods for such derivatization.

**[0087]** Fluticasone—Fluticasone propionate is a synthetic corticosteroid. Fluticasone propionate is a white to off-white powder and is practically insoluble in water, freely soluble in dimethyl sulfoxide and dimethylformamide, and slightly soluble in methanol and 95% ethanol. In an embodiment, the formulations of the present invention may comprise a steroidal anti-inflammatory (e.g., fluticasone propionate).

**[0088]** Beclomethasone—In certain aspects the steroidal anti-inflammatory can be beclomethasone dipropionate or its monohydrate. The compound may be a white powder and is very slightly soluble in water (Physicians' Desk Reference), very soluble in chloroform, and freely soluble in acetone and in alcohol.

**[0089]** Providing steroidal anti-inflammatories according to the present invention may enhance the compositions and methods of the invention by, for example, attenuating any unwanted inflammation. Examples of other steroidal anti-inflammatories for use herein include, but are not limited to, betamethasone, triamcinolone, dexamethasone, prednisone, mometasone, flunisolide and budesonide.

**[0090]** In accordance with yet another aspect of the invention, the non-steroidal anti-inflammatory agent may include aspirin, sodium salicylate, acetaminophen, phenacetin, ibuprofen, ketoprofen, indomethacin, flurbiprofen, diclofenac, naproxen, piroxicam, tebufelone, etodolac, nabumetone, tenidap, alcofenac, antipyrine, amimopyrine, dipyrrone, ammpyrone, phenylbutazone, clofezone, oxyphenbutazone, prexazone, apazone, benzydamine, bucolome, cinchopen, clonixin, ditrazol, eprizole, fenoprofen, floc-tafeninl, flufenamic acid, glaphenine, indoprofen, meclofenamic acid, mefenamic acid, niflumic acid, salidif-amides, sulindac, suprofen, tolmetin, nabumetone, tiar-amide, proquazone, bufexamac, flumizole, tinoridine, tim-egadine, dapsone, diflunisal, benorylate, fosfosal, fenclofenac, etodolac, fentiazac, tilomisolet, carprofen, fenbufen, oxaprozin, tiaprofenic acid, pirofen, feprazone, piroxicam, sudoxicam, isoxicam, celecoxib, Vioxx®, and/or tenoxicam.

#### IV. Kits

**[0091]** Any of the compositions described herein may be comprised in a kit. In a nonlimiting example, reagents for production and/or delivery of a therapeutic composition described herein are included in a kit. In certain aspects the kit is portable and may be carried on a person much like an asthma inhaler is carried. The kit may further include a pathogen detector. The kit may also contain a gas or mechanical propellant for compositions of the invention.

**[0092]** The components of the kits may be packaged either in an aqueous, powdered, or lyophilized form. The container means of the kits will generally include at least one inhaler, canister, vial, test tube, flask, bottle, syringe or other container means, into which a component(s) may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit (second agent, etc.), the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial, canister, or inhaler. A container of the invention can include a canister or inhaler that can be worn on a belt or easily carried in a pocket, backpack or other storage container. The kits of the present invention also will typically include a container for the described compositions or their variations, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow molded plastic containers into which the desired vials are retained.

**[0093]** When the components of the kit are provided in one and/or more liquid solutions, e.g., the liquid solution is an aqueous solution, with a sterile aqueous solution being particularly preferred, but not required. However, the components of the kit may be provided as dried powder(s). When reagents and/or components are provided as a dry powder, the powder may be reconstituted by the addition of a suitable solvent or administered in a powdered form. It is envisioned that a solvent may also be provided in another container.

**[0094]** A kit will also include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions may include variations that can be implemented.

**[0095]** It is contemplated that such reagents are embodiments of kits of the invention. Such kits, however, are not limited to the particular items identified above and may include any reagent used directly or indirectly in the detection of pathogenic microorganisms or administration of a composition described herein.

**[0096]** The inventors have used the mouse as model for microbial infection of the lung. Not be held to any particular mechanism or theory, it is believed that the increase in resistance to infection is due to activation of local defenses or innate immunity. The effects of single and repetitive exposure of a subject to a composition of the invention have been determined and no obvious gross pathology, such as premature death, weight loss, or behavioral changes have been observed.

**[0097]** One non-limiting benefit of the present invention is that it can be delivered and have effect quickly and easily. Also, the compositions can be produced economically in large quantities and easily stored, as well as easily transported by a person outside of a hospital setting. Typically, the administration of the inventive compositions and the methods of the invention result in at least some killing or inhibition of the invading pathogens even before cellular entry. In the case that some pathogens do enter cells in the lungs either by escaping extracellular killing or because the compositions are administered after pathogen exposure (pre-emptively) instead of before pathogen exposure (preventatively), it is contemplated that the compositions and related methods promote intracellular killing resulting from the enhanced or augmented local responses in the lungs.

**[0098]** A composition described in this application would simplify countermeasure stockpiling and deployment. Also,

the compositions and methods of the invention would eliminate the difficulty of rapidly identifying a specific pathogen during a bioweapon attack or other exposure or potential exposure event. In addition, the economic advantages of producing and purchasing an agent with applicability in multiple civilian and biodefense settings are significant. Augmenting local epithelial mechanisms is particularly attractive in subjects who often have neutropenia or impaired adaptive immune function, e.g., immune compromised subjects. The methods typically act locally rather than systemically, and provide broad effects against multiple pathogens. The effects are rapid and are attractive in a biodefense, medical, and epidemic setting. Augmentation of innate defense capabilities of the lungs in normal hosts would be valuable during influenza or emergent respiratory viral epidemics for which adaptive immune vaccines are not available. Similarly, protection of caregivers during an epidemic would facilitate care of the sick while limiting spread.

**[0099]** Many people in the community live with chronically compromised defenses against infection, such as patients with diabetes and patients taking immunosuppressive drugs for autoimmune diseases or to prevent transplant rejection. These people will benefit from a treatment or an increased resistance to infection during epidemics or times where potential for exposure to viruses is elevated. Even more strikingly, cancer patients undergoing chemotherapy who have transient but severely compromised immune defenses might benefit from transient protection. Pneumonia is a common occurrence in these patients, and is the leading cause of infectious death.

**[0100]** Resistance to infection can be stimulated to provide transient protection during prolonged periods of neutropenia. Other cancer patients, such as those receiving fludarabine or anti-lymphocyte antibodies, or those receiving calcineurin inhibitors and steroids after hematopoietic stem cell transplantation, have impaired adaptive immunity. These patients might also benefit from episodic stimulation of immunity to protect against epidemic viruses. Community outbreaks of seasonal respiratory viruses such as influenza, parainfluenza, and RSV can cause fatal pneumonia in these compromised patients, and infection with many of these viruses can be rapidly identified from nasal washings.

## V. Viruses

**[0101]** Class A bioterrorism agents that can be transmitted by aerosol include smallpox virus, and hemorrhagic fever viruses. Class B and class C bioterrorism agents also can be effectively delivered by the respiratory route. These organisms comprise a variety of viral classes. Because of the potential difficulty in initially identifying a specific agent, the complexity of locally stockpiling adaptive immune vaccines and antibiotics directed at specific agents, and the remarkable virulence of organisms despite appropriate treatment, stimulation of innate defense capabilities and increasing the resistance of the lungs to infection can prevent or preempt or attenuate infection with an agent delivered by the respiratory route. Such an effect could have great public health value.

**[0102]** There are numerous microbes that are considered pathogenic or potentially pathogenic under certain conditions (i.e., opportunistic pathogens/microbes). In certain aspects, the pathogenicity is determined relative to infection via the lungs. In certain aspects the microbe is a virus. There

are numerous viruses and viral strains that are considered pathogenic or potentially pathogenic under certain conditions.

**[0103]** Viruses can be placed in one of the seven following groups: Group I: double-stranded DNA viruses, Group II: single-stranded DNA viruses, Group III: double-stranded RNA viruses, Group IV: positive-sense single-stranded RNA viruses, Group V: negative-sense single-stranded RNA viruses, Group VI: reverse transcribing Diploid single-stranded RNA viruses, Group VII: reverse transcribing Circular double-stranded DNA viruses. Viruses include the family Adenoviridae, Arenaviridae, Caliciviridae, Coronaviridae, Filoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae (Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae), Nidovirales, Papillomaviridae, Paramyxoviridae (Paramyxovirinae, Pneumovirinae), Parvoviridae (Parvovirinae, Picornaviridae), Poxviridae (Chordopoxvirinae), Reoviridae, Retroviridae (Orthoretrovirinae), and/or Togaviridae. These viruses include, but are not limited to various strains of influenza, such as avian flu (e.g., H5N1). Particular virus from which a subject may be protected include, but is not limited to Cytomegalovirus, Respiratory syncytial virus and the like.

**[0104]** Examples of pathogenic viruses include, but are not limited to Influenza A, H5N1, Marburg, Ebola, Dengue, Severe acute respiratory syndrome coronavirus, Yellow fever virus, Human respiratory syncytial virus, Vaccinia virus and the like.

## VI. Formulations and Administration

**[0105]** The pharmaceutical compositions disclosed herein may be administered via the respiratory system of a subject. In certain aspects the compositions are deposited in the lung by methods and devices known in the art. Therapeutic compositions described herein may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof, and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for inhalation include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile inhalable solutions or dispersions. In all cases the form is typically sterile and capable of inhalation directly or through some intermediary process or device. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

**[0106]** Some variation in dosage will necessarily occur depending on the condition of the subject being treated and the particular circumstances involving exposure or potential exposure. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety, and purity

standards as required by FDA Office of Biologics standards or other similar organizations.

**[0107]** Sterile compositions are prepared by incorporating the active components in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by, for example, filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile compositions, some methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the component(s) and/or active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution.

**[0108]** Pulmonary/respiratory drug delivery can be implemented by different approaches, including liquid nebulizers, aerosol-based metered dose inhalers (MDI's), sprayers, dry powder dispersion devices and the like. Such methods and compositions are well known to those of skill in the art, as indicated by U.S. Pat. Nos. 6,797,258, 6,794,357, 6,737,045, and 6,488,953, all of which are incorporated by reference. According to the invention, at least one pharmaceutical composition can be delivered by any of a variety of inhalation or nasal devices known in the art for administration of a therapeutic agent by inhalation. Other devices suitable for directing pulmonary or nasal administration are also known in the art. Typically, for pulmonary administration, at least one pharmaceutical composition is delivered in a particle size effective for reaching the lower airways of the lung or sinuses. Some specific examples of commercially available inhalation devices suitable for the practice of this invention are Turbohaler™ (Astra), Rotahaler® (Glaxo), Diskus® (Glaxo), Spiros™ inhaler (Dura), devices marketed by Inhale Therapeutics, AERx™ (Aradigm), the Ultravent® nebulizer (Mallinckrodt), the Acorn II® nebulizer (Marquest Medical Products), the Ventolin® metered dose inhaler (Glaxo), the Spinhaler® powder inhaler (Fisons), Aerotech II® or the like.

**[0109]** All such inhalation devices can be used for the administration of a pharmaceutical composition in an aerosol. Such aerosols may comprise either solutions (both aqueous and non-aqueous) or solid particles. Metered dose inhalers typically use a propellant gas and require actuation during inspiration. See, e.g., WO 98/35888 and WO 94/16970. Dry powder inhalers use breath-actuation of a mixed powder. See U.S. Pat. Nos. 5,458,135 and 4,668,218; PCT publications WO 97/25086, WO 94/08552 and WO 94/06498; and European application EP 0237507, each of which is incorporated herein by reference in their entirety. Nebulizers produce aerosols from solutions, while metered dose inhalers, dry powder inhalers, and the like generate small particle aerosols. Suitable formulations for administration include, but are not limited to nasal spray or nasal drops, and may include aqueous or oily solutions of a composition described herein.

**[0110]** A spray comprising a pharmaceutical composition described herein can be produced by forcing a suspension or solution of a composition through a nozzle under pressure. The nozzle size and configuration, the applied pressure, and the liquid feed rate can be chosen to achieve the desired

output and particle size. An electrospray can be produced, for example, by an electric field in connection with a capillary or nozzle feed.

**[0111]** A pharmaceutical composition described herein can be administered by a nebulizer such as a jet nebulizer or an ultrasonic nebulizer. Typically, in a jet nebulizer, a compressed air source is used to create a high-velocity air jet through an orifice. As the gas expands beyond the nozzle, a low-pressure region is created, which draws a composition through a capillary tube connected to a liquid reservoir. The liquid stream from the capillary tube is sheared into unstable filaments and droplets as it exits the tube, creating the aerosol. A range of configurations, flow rates, and baffle types can be employed to achieve the desired performance characteristics from a given jet nebulizer.

**[0112]** In an ultrasonic nebulizer, high-frequency electrical energy is used to create vibrational, mechanical energy, typically employing a piezoelectric transducer. This energy is transmitted to the composition creating an aerosol.

**[0113]** In a metered dose inhaler (MDI) or in other device that uses propellant, a propellant, a composition, and any excipients or other additives are contained in a canister as a mixture with a compressed gas. Actuation of the metering valve releases the mixture as an aerosol. Pharmaceutical compositions for use with a metered-dose inhaler device will generally include a finely divided powder containing a composition of the invention as a suspension in a non-aqueous medium, for example, suspended in a propellant with the aid of a surfactant. The propellant can be any conventional material employed for this purpose such as chlorofluorocarbon, a hydrochlorofluorocarbon, a hydrofluorocarbon, or a hydrocarbon including trichlorofluoromethane, dichlorodifluoromethane, dichlorotetrafluoroethanol and 1,1,1,2-tetrafluoroethane, HFA-134a (hydrofluoroalkane-134a), HFA-227 (hydrofluoroalkane-227), or the like.

**[0114]** As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

**[0115]** The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a subject. The preparation of an aqueous composition that contains a polypeptide or peptide as an active ingredient is well understood in the art.

## VII. Examples

**[0116]** The following examples as well as the figures are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples or figures represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and

still obtain a like or similar result without departing from the spirit and scope of the invention.

### Example 1

#### Effect of Time and Frequency of PUL042 and Combination Treatments Against Influenza

**[0117]** Studies were designed and performed to compare the effect of timing and frequency of aerosolized PUL042 (Pam2CSK4+ODN-M362) and oral or aerosolized Tamiflu (Osetamivir phosphate) as combination treatments to inhibit pulmonary influenza A/HK/8/68 (H3N2) virus (FluA) infection in mice. Survival and body weights were followed up to 21 days. Similar studies were also conducted using the antiviral ribavirin (RBV). (See FIG. 1, FIG. 2, FIG. 3, and FIG. 4). Similar studies have been conducted with coronavirus such as MERS-COV and SARS-COV (See FIG. 5, FIG. 6, and FIG. 7).

**[0118]** Mice: NIH Swiss-Webster, female, 6-8 weeks of age, approximately 20 g. On day 0 hour 0 mice are divided into groups and infected. Treats start at +48 h (day 2). Groups included the following:

**[0119]** Group 1: Untreated, infected control, no treatments.

**[0120]** Group 2: Water by gavage at +48, +72 and +96 h (no infection)

**[0121]** Group 3: Tamiflu by gavage at 4 mg/kg/day given at +48, +72 and +96 h

**[0122]** Group 4: Aerosol PUL042 at +48 h.

**[0123]** Group 5: Aerosol PUL042 at +48 and +96 h.

**[0124]** Group 6: Aerosol PUL042 plus Tamiflu by gavage at +48 h followed by Tamiflu by gavage at +72 and +96 h.

**[0125]** Group 7: Aerosol PUL042 plus Tamiflu by gavage at +48 and +96 h with Tamiflu by gavage at +72 h.

**[0126]** Group 8: Aerosol PUL042/Tamiflu combination at +48 h.

**[0127]** Group 9: Aerosol PUL042/Tamiflu combination at +48 and +96 h with aerosol Tamiflu at +72 h.

**[0128]** Group 10: aerosol Tamiflu at +48, +72 and +96 h

**[0129]** PUL-042: 16  $\mu$ M Pam2CSK4+4  $\mu$ M ODN-M362 formulated and supplied by Pulmotect for each aerosol exposure. Five (5) mL for 15 min aerosol treatments are needed (total for 3 exposures=20 mL of PUL042).

**[0130]** Virus: Influenza A/HK/8/68 (H3N2; Mouse Lung Pool Jan. 17, 2012). Stock titer=7.64  $\log_{10}$  TCID<sub>50</sub>/mL. Dilute virus 1:500 in 0.05% gelatin-MEM [1:500=20.0  $\square$  to 10 mL of 0.05% gelatin-MEM]; use 9 mL in reservoir of nebulizer. Titer is determined in pre- and post-nebulization reservoir solutions. Estimated virus/mouse when exposed to aerosol for 20 min with Aerotech II nebulizer flowing at 10 L/min air  $\sim 10^5$  TCID<sub>50</sub>.

**[0131]** Virus infection: Half of the mice from each group is randomized into 1 of 2 treatment boxes and exposed to influenza virus aerosol for 20 min. Virus and drug exposures are generated from an Aerotech II nebulizer flowing at 10 L/min of room air generated from an Aridyne 2000 compressor.

**[0132]** PUL042 treatments: Mice are placed into a sealed plastic box. For PUL042 treatment, a selected group of mice is administered aerosol of PUL042 for 15 min. After exposure, mice are returned to their pre-assigned groups.

**[0133]** Oral Tamiflu (oseltamivir phosphate) gavage treatments: Osetamivir phosphate is obtained from Tamiflu capsules. For gavage, powder from 1 capsule (163 mg/cap-

sule; 45% oseltamivir carboxylate equivalent) is suspended in 1 mL of sterile water, vortexed, and sonicated in a water bath at room temperature for 1-5 min. The solution is equivalent to 75 mg oseltamivir carboxylate/mL. For each treatment, Tamiflu is diluted and administered by gavage (oral) using 100  $\mu$ L of 0.8 mg Osetamivir carboxylate/mL (dilute: 0.424 mL of 75 mg Osel/mL+39.576 mL H<sub>2</sub>O) for a dose of 4 mg/kg/day in 100  $\mu$ L.

**[0134]** Aerosols of PUL042 and/or Tamiflu: Osetamivir phosphate is obtained from Tamiflu capsules. For each day's aerosol, powder from 5 or 6 capsules (167 $\pm$ 1 mg/capsule; 45.0% oseltamivir carboxylate equivalent) is suspended in 5 or 6 mL of either PUL042 (combination) or in sterile water (Tamiflu-only) and vortexed vigorously. The suspension is centrifuged at full speed in the clinical centrifuge for 15 min and the supernatant fraction is removed and place in the nebulizer. The solution is equivalent to 75 mg oseltamivir carboxylate/mL. The estimated deposition is 1.7 mg/kg in the lungs and 3.4 mg/kg in the stomach.

**[0135]** Procedures: On Day 0, all Groups are exposed to a 20 min aerosol calculated to deposit approximately 10<sup>5</sup> TCID<sub>50</sub> of FluA/HK per mouse (approximately 85-100% mortality). Mice are returned to their appropriate groups and weighed.

**[0136]** On Day +2 (+48 h), Group 1 is treated with oral water; Groups 4, 5, 6, and 7 are treated with aerosolized PUL042; Groups 3, 6, and 7 are treated with Oral Tamiflu; Groups 8 and 9 are treated with a combination of PUL042 and Tamiflu; Group 10 is treated with aerosolized Tamiflu.

**[0137]** On Day +3 (+72 h), Group 1 is treated with oral water; Groups 3, 6, and 7 are treated with oral Tamiflu; and Groups 9 and 10 are treated with aerosolized Tamiflu.

**[0138]** On Day +4 (+96 h), Group 1 is treated with oral water; Groups 5 and 7 are treated with aerosolized PUL042; Groups 3, 6, and 7 are treated with Oral Tamiflu; Group 9 is treated with combination PUL042 and Tamiflu; and Group 10 is treated with aerosolized Tamiflu.

**[0139]** Mice in each group are observed daily for overt illness, morbidity, and mortality. Mice are weighed on Days 0, 4 through 11; and days 14 to 21, if necessary.

#### Protocol:

##### [0140]

| Group <sup>1</sup> | Aerosol Dose:                            | Duration Aerosol (min) | Oral Dose (mg/kg/day) q.d. | Drug Treatment (day) | FluA Challenge Day 0 | End-points             |
|--------------------|------------------------------------------|------------------------|----------------------------|----------------------|----------------------|------------------------|
| 1                  | 0                                        | None                   | 0                          | None                 | Yes                  | Body weights; Survival |
| 2                  | 214 + 266                                | 15                     | —                          | +2, 3, 4             | No                   |                        |
| 3                  | ng/kg/day <sup>2</sup>                   | 15                     | 4                          | +2, 3, 4             | Yes                  |                        |
| 4                  |                                          | 15                     | —                          | +2                   | Yes                  |                        |
| 5                  |                                          | 15                     | —                          | +2, 4                | Yes                  |                        |
| 6                  |                                          | 15                     | 4                          | +2, 3, 4             | Yes                  |                        |
| 7                  |                                          | 15                     | 4                          | +2, 3, 4             | Yes                  |                        |
| 8                  | 214 + 266                                | 15                     | —                          | +2                   | Yes                  |                        |
| 9                  | ng/kg/day <sup>2</sup> ;                 | 15                     | —                          | +2, 3, 4             | Yes                  |                        |
| 10                 | L: 1.7<br>S: 3.4<br>mg/kg/d <sup>3</sup> | 15                     | —                          | +2, 3, 4             | Yes                  |                        |

<sup>1</sup>Mice, 15/group; <sup>2</sup>Estimated deposited dosage of Pam2CSK4 + ODN-M362, 16  $\mu$ M Pam2 + 4  $\mu$ M ODN; <sup>3</sup>Estimated deposited dosage (mg/kg/day) of aerosolized Osetamivir (75 mg/mL) in lungs (L) and stomach (S).

Abbreviations:

Rx = treatment; Combo, combination PUL042 aerosol + oral or aerosolized Tamiflu; FluA = influenza A/HK/8/68 (H3N2) virus.

## Timing of Treatments:

## [0141]

| Group | Day 0 | Day 1 | Day 2     | Day 3   | Day 4     | Total Rx (days) |
|-------|-------|-------|-----------|---------|-----------|-----------------|
| 1     | Virus | —     | —         | —       | —         | None            |
| 2     | Virus | —     | Water-g   | Water-g | Water-g   | 3               |
| 3     | Virus | —     | O-g       | O-g     | O-g       | 3               |
| 4     | Virus | —     | P         | —       | —         | 1               |
| 5     | Virus | —     | P         | —       | P         | 2               |
| 6     | Virus | —     | P + O-g   | O-g     | O-g       | 3               |
| 7     | Virus | —     | P + O-g   | O-g     | P + O-g   | 3               |
| 8     | Virus | —     | P + O-aer | —       | —         | 1               |
| 9     | Virus | —     | P + O-aer | O-aer   | P + O-aer | 3               |
| 10    | Virus | —     | O-aer     | O-aer   | O-aer     | 3               |

P + O-g or P + O-aer = Combo Rx, PUL042 aerosol + Oral or aerosolized Tamiflu; O-g = Tamiflu Oral only; O-aer, Tamiflu Aerosol only.

Abbreviations:

H, water; O, Oseltamivir as Tamiflu; P, PUL042; g, gavage; a, aerosol; D, day of treatment

## Example 2

### Dose-Response and Temporal Efficacy of Aerosolized PUL-042 Administered in Combination with Ribavirin in Attenuating RSV Infection

[0142] RSV is a major cause of pneumonia and bronchiolitis in infants, the elderly, and immunocompromised transplant patients, and is a major cause of respiratory infection leading to asthma exacerbations. Further, an immune-suppressed model has been described in which cotton rats (CR) treated with cyclophosphamide exhibit characteristics of persistent RSV infection. These conditions are physiologically relevant to studies targeting immune-suppressed populations at risk for RSV infections. In addition, more than 200 CR genes have been cloned encoding cytokines, chemokines, and lymphocyte cell surface markers. Analysis of these genes can inform mechanisms of viral pathogenesis and clearance in the presence or absence of therapeutic treatments.

[0143] Using aerosolized forms of both drugs given alone or in combination, the comparative effects of a single dose versus two doses, and of treatment begun early after infection, or relatively late in the course of infection is evaluated.

[0144] The studies use an established animal model for RSV infection, the cotton rat, to evaluate the ability of addition of PUL-042 to ribavirin treatment to inhibit viral infection and replication in the nasopharyngeal compartment and compare this to the activity in the lung, which is more representative of later stage or more severe viral disease.

[0145] Cotton rats are the optimal model for these studies because they are 100-fold more permissive than mice to RSV infections in both the upper and lower airways, and infected animals develop pathology similar to that seen in humans. The predictive quality of the CR model for therapeutics in treating RSV infections advanced clinical trials of RSVIg, Respigam and palivizumab, and an effective protocol for Ribavirin treatment is well established in the cotton rat.

## Experimental Methods:

[0146] RSV inoculation and PUL-042 drug delivery: *Sigmodon hispidus* cotton rats (CR) are ~75-150 g body weight as determined by the age at start of the experiment. Animal

body weight and sex distribution is as similar as possible across all groups at the start. Body weights are recorded at end of the experiment. RSV/A/Tracy,  $1.22 \times 10^5$  PFU is given to CR lightly anesthetized with isoflurane. For PUL-042 or ribavirin treatment, CR are placed into a sealed plastic box. PUL-042 and ribavirin exposures are generated from a Pari LC Sprint nebulizer flowing at 10 L/min of room air generated from a compressor.

[0147] Lung and nasal tissue homogenates and histopathology: Following CO<sub>2</sub> euthanasia, for the same animal, one lung lobe is clamped off for organ homogenate and the remaining lobes are perfused with 10% neutral buffered formalin, and inflated for paraffin embedding. To evaluate the upper airways, one nasal turbinate is prepared for histopathology and the other is used to prepare tissue homogenates. Plaque assays are performed on the lung and nasal homogenates. Total RNA is extracted from lung and nasopharyngeal tissues and the kinetics of RSV genome replication is measured by RT-qPCR. This total lung RNA may also be used to evaluate expression of cotton rat genes associated with pathogenesis of RSV disease.

[0148] Histopathology: Intact lung tissue from the formalin-fixed lobes is prepared for histology. Sections are stained with hematoxylin-eosin and coded for blinded scoring of histopathology by veterinary pathologists. Sections are scored from 0 to 4 based on the extent and severity of alveolitis, alveolar eosinophilia, bronchiolitis, bronchiolar eosinophilia, peribronchiolar mononuclear inflammatory cell infiltrates, and perivascular mononuclear inflammatory cell infiltrates.

[0149] In these experiments, PUL-042 and ribavirin are at the concentrations (nebulized in 5 mL water as ribavirin at 100 mg/mL; PUL-042 at 17 µg/mL ODN+11.6 µg/mL PAM2) applied in prior mouse influenza A experiments. All experiments are repeated once for confirmation of results.

[0150] Evaluate the optimal start of treatment and interval of treatment. In prior experience with PUL-042 combined with ribavirin against influenza A, it was found that two sequential combination treatments on days 1 and 2 post-infection resulted in a 92% increased survival rate, whereas ribavirin alone on those two days provided only minimal improvement in survival rate, at 15%, compared to 0% of untreated. The greatest efficacy of PUL-042 as a monotherapy was found when the drug was administered on Day 1 post-infection (approximately 40% survival) with the benefit dropping rapidly if treatment was further delayed.

[0151] Dose Schedules of PUL-042=/~~—~~Ribavirin in RSV-infected cotton rats.

| Group       | RSV      | PUL-042   | RBV       | Combina-<br>tion | Viral Titer<br>Evaluation |
|-------------|----------|-----------|-----------|------------------|---------------------------|
| Control 1   | Infected | UT        | UT        | UT               | D 4                       |
| Control 2   | Infected | UT        | UT        | UT               | D 5                       |
| Control 3   | Infected | D - 1     | UT        | UT               | D 4                       |
| Control 4   | Infected | D 1 + D 2 | UT        | UT               | D 4                       |
| Control 5   | Infected | UT        | D 1 + D 2 | UT               | D 4                       |
| Treatment 1 | Infected | UT        | UT        | D 1 + D 2        | D 4                       |
| Treatment 2 | Infected | UT        | UT        | D 1 + D 3        | D 4                       |
| Treatment 3 | Infected | UT        | UT        | D 1 + D 4        | D 5                       |
| Treatment 4 | Infected | UT        | UT        | D 2 + D 3        | D 4                       |
| Treatment 5 | Infected | UT        | UT        | D 2 + D 4        | D 5                       |

UT = Untreated;

D - 1 = 24 h before infection;

D1 = Day 1 post-infection

[0152] The RSV infection in CR is not lethal. Demonstration of an effect of PUL-042 and ribavirin for RSV requires measurement of viral load during the course of infection and clearance, and evaluation of histopathology during the course of RSV disease in the same animals. The effect of two doses of PUL-042 and ribavirin initiated on day 1 post-infection, followed by a second treatment on Day 2, or Day 3, or Day 4 and those initiated on day 2 post-infection, followed by treatment on Day 3 or Day 4 is evaluated. For treatment schedules ending before Day 4, animals are euthanized for analysis on Day 4. In addition to untreated controls for evaluation on days 4 and 5, ribavirin is evaluated alone administered on Day 1 and Day 2 with evaluation of Day 4 titers. Because the day of maximal proliferation is Day 4, and RSV is cleared in these animals by Day 7, any treatment occurring after Day 4 may not be distinguishable from the result in untreated animals.

[0153] Simulation of treatment in an immune-suppressed patient population. The optimal time course and dosing

schedule can be repeated in cotton rats undergoing cyclophosphamide (CY) treatment and the effect of PUL-042 alone or combined with ribavirin is measured. As previously described, intraperitoneal dosing of CY maintains a state of leukopenia in cotton rats without affecting mortality. RSV infection in CY-treated CR is persistent as shown by prolonged high titers in lung tissue at 12 days post-infection. CR are given CY intraperitoneal (i.p.) injections of 50 mg/kg three times per week for 3 weeks before RSV infection, and continues until the end of the time course for each animal. Immunosuppression is confirmed by complete blood counts (CBC) in blood collected at necropsy by cardiac puncture from CY-treated and untreated control animals. In addition to the day 4 time point for measuring virus titers, titers are measured in the CY-immune suppressed animals at day 10 to confirm persistent RSV infection and to determine what effect PUL-042 has on virus replication later in infection. Serum cytokines levels are also measured in the blood samples.

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13

1. A composition comprising: (a) lipopeptide, (b) immune stimulatory oligodeoxy nucleotide, and (c) antiviral pharmaceutical.

2. The composition of claim 1, wherein the lipopeptide is PAM<sub>2</sub>CSK<sub>4</sub>.

3. The composition of claim 1, wherein the immune stimulatory oligodeoxynucleotide is a type C oligodeoxynucleotide (ODN).

4. The composition of claim 3, wherein the immune stimulatory oligodeoxynucleotide is ODN2395 or ODNM362 or ODN10101.

5. The composition of claim 1, wherein the antiviral pharmaceutical is oseltamivir or ribavirin.

6. The composition of claim 1, wherein the composition is formulated for administration to the lungs.

7. A method of treating, inhibiting, or attenuating a viral infection comprising administering an effective amount of the composition of claim 1 to an individual that has or is at risk of viral infection.

8. The method of claim 7, wherein the subject has been exposed to a virus.

9. The method of claim 7, wherein the virus is a Adenoviridae, Coronaviridae, Filoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Paramyxovirinae, Pneumovirinae, Picomaviridae, Poxviridae, Retroviridae, or Togaviridae.

10. The method of claim 7, wherein the virus is Parainfluenza, Influenza, H5N1, Marburg, Ebola, Severe acute respiratory syndrome coronavirus (SARS-COV), Middle eastern respiratory syndrome coronavirus (MERS-COV), yellow fever virus, human respiratory syncytial, Hantavirus, or Vaccinia virus.

11. The method of claim 7, wherein the composition is administered by nebulization.

12. The method of claim 7, wherein the lipopeptide, immune stimulatory oligonucleotide and anti-viral drug are administered in an amount of from about 0.1 mg/kg to about 100 mg/kg of the individual's body weight.

13. A method of treating, inhibiting, or attenuating a viral infection comprising administering an effective amount of the composition comprising an aerosolized (a) lipopeptide and (b) immune stimulatory oligodeoxy nucleotide in combination with a composition comprising an anti-viral pharmaceutical to an individual that has or is at risk of viral infection.

14. The method of claim 13, wherein the lipopeptide is PAM<sub>2</sub>CSK<sub>4</sub> and wherein the immune stimulatory oligodeoxynucleotide is ODN2395 or ODNM362 or ODN10101.

15. The method of claim 13, wherein the immune stimulatory oligodeoxynucleotide is a type C oligodeoxynucleotide (ODN).

16. (canceled)

17. The method of claim 13, wherein the antiviral pharmaceutical is oseltamivir or ribavirin.

18-19. (canceled)

20. The composition of claim 1, wherein the composition is formulated for nebulization.

21. A nebulizer comprising (a) lipopeptide, (b) immune stimulatory oligodeoxy nucleotide, and (c) anti-viral pharmaceutical.

22-25. (canceled)

26. The composition of claim 1, wherein the composition comprises 16  $\mu$ M lipopeptide and 4  $\mu$ M immune stimulatory oligodeoxy nucleotide.

27. The composition of claim 1, wherein the molar ratio of lipopeptide to immune stimulatory oligodeoxy nucleotide is 4:1.

\* \* \* \* \*