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(54) Title: OLIGORIBONUCLEOTIDES AND USES THEREOF

Figure 1A

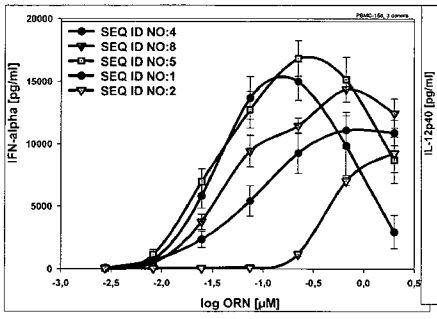


Figure 1B

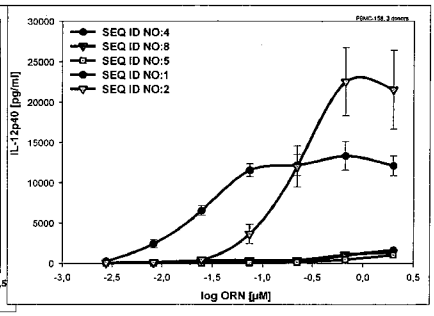


Figure 1

(57) Abstract: The invention relates to immunostimulatory RNA oligonucleotides (ORN). In particular the ORN have an immunostimulatory ORN motif directly or indirectly flanked by a 3' poly G motif and optionally a 5' poly-G motif. The invention also relates to methods including therapeutic methods and screening methods and related kits for use of the ORN.

WO 2008/139262 A2

OLIGORIBONUCLEOTIDES AND USES THEREOF

BACKGROUND OF INVENTION

1. Field of Invention

The invention relates to immunostimulatory RNA oligonucleotides (ORN). In particular the ORN have an immunostimulatory ORN motif directly or indirectly flanked by a 3' poly G motif and optionally a 5' poly-G motif. The invention also relates to methods including therapeutic methods and screening methods and related kits for use of the ORN.

2. Background

Toll-like receptors (TLRs) are a family of highly conserved pattern recognition receptor (PRR) polypeptides that recognize pathogen-associated molecular patterns (PAMPs) and play a critical role in innate immunity in mammals. Currently at least ten family members, designated TLR1 - TLR10, have been identified. The cytoplasmic domains of the various TLRs are characterized by a Toll-interleukin 1 receptor (TIR) domain. Medzhitov R et al. (1998) *Mol Cell* 2:253-8. Recognition of microbial invasion by TLRs triggers activation of a signaling cascade that is evolutionarily conserved in *Drosophila* and mammals. The TIR domain-containing adapter protein MyD88 has been reported to associate with TLRs and to recruit interleukin 1 receptor-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) to the TLRs. The MyD88-dependent signaling pathway is believed to lead to activation of NF-κB transcription factors and c-Jun NH_2 terminal kinase (Jnk) mitogen-activated protein kinases (MAPKs), critical steps in immune activation and production of inflammatory cytokines. For reviews, see Aderem A et al. (2000) *Nature* 406:782-87, and Akira S et al. (2004) *Nat Rev Immunol* 4:499-511.

A number of specific TLR ligands have been identified. Ligands for TLR2 include peptidoglycan and lipopeptides. Yoshimura A et al. (1999) *J Immunol* 163:1-5; Yoshimura A et al. (1999) *J Immunol* 163:1-5; Aliprantis AO et al. (1999) *Science* 285:736-9. Lipopolysaccharide (LPS) is a ligand for TLR4. Poltorak A et al. (1998) *Science* 282:2085-8; Hoshino K et al. (1999) *J Immunol* 162:3749-52. Bacterial flagellin is a ligand for TLR5. Hayashi F et al. (2001) *Nature* 410:1099-1103. Peptidoglycan has been reported to be a ligand not only for TLR2 but also for TLR6. Ozinsky A et al.

(2000) *Proc Natl Acad Sci USA* 97:13766-71; Takeuchi O et al. (2001) *Int Immunol* 13:933-40. Recently certain low molecular weight synthetic compounds, the imidazoquinolines imiquimod (R-837) and resiquimod (R-848), were reported to be ligands of TLR7 and TLR8. Hemmi H et al. (2002) *Nat Immunol* 3:196-200; Jurk M et al. (2002) *Nat Immunol* 3:499.

Beginning with the recent discovery that unmethylated bacterial DNA and synthetic analogs thereof (CpG DNA) are ligands for TLR9 (Hemmi H et al. (2000) *Nature* 408:740-5; Bauer S et al. (2001) *Proc Natl Acad Sci USA* 98, 9237-42), it has been reported that ligands for certain TLRs include certain nucleic acid molecules. Recently it has been reported that certain types of RNA are immunostimulatory in a sequence-independent or sequence-dependent manner. Further, it has been reported that these various immunostimulatory RNAs stimulate TLR3, TLR7, and TLR8.

SUMMARY OF INVENTION

The invention relates generally to immunostimulatory oligoribonucleotides (ORN) that contain certain RNA motifs, as well as to related immunostimulatory compositions containing such ORN, and methods for the use of such ORN and compositions. The ORN of the invention may be useful in any setting or application that calls for stimulating or augmenting an immune response. As disclosed below, the ORN of the invention are of particular use in the preparation of pharmaceutical compositions, including adjuvants, vaccines, and other medicaments, for use in treating a variety of conditions, including infection, cancer, allergy, and asthma. The invention in certain aspects thus relates to compositions that include ORN of the invention, as well as methods of their use. Also as disclosed below, the ORN and compositions of the invention are of particular use in methods for activating an immune cell, vaccinating a subject, treating a subject having an immune system deficiency, treating a subject having an infection, treating a subject having autoimmune disease, treating a subject having cancer, treating a subject having an allergic condition, treating a subject having asthma, airway remodeling, promoting epitope spreading, and antibody-dependent cellular cytotoxicity (ADCC).

As disclosed in greater detail below, the immunostimulatory ORN of the invention are characterized by their inclusion of at least one sequence-dependent

immunostimulatory RNA motif. The sequence-dependent immunostimulatory RNA motif generally is a short RNA sequence, although in certain embodiments the motif can also include one or more modifications such as a modified internucleotide phosphate linkage, a modified nucleobase, a modified sugar, a nucleotide analog, deoxyribonucleotide, a spacer, a non-nucleotidic linker or any combination thereof. In one embodiment the immunostimulatory RNA motif occurs in the context of a longer immunostimulatory ORN of the invention. Also the immunostimulatory RNA motif may occur in the context of a chimeric DNA:RNA nucleic acid molecule.

The sequence-dependent immunostimulatory RNA motifs and ORN incorporating such motifs are disclosed to be agonists for TLR7 but not TLR8. An immunostimulatory RNA oligonucleotide (ORN) of 8-100 ribonucleotides in length is provided according to some aspects of the invention. The ORN includes an immunostimulatory ORN motif linked to a poly-G motif, wherein the poly-G motif is 3' to the immunostimulatory ORN motif. The poly-G motif comprises at least 4 Gs. The poly G motif may be 5, 6, 7, 8, 9, or 10 G's in other embodiments. G is guanosine or a derivative thereof.

In some embodiments the ORN is not one of the following 5' GGGGUUUUGGGGG 3' (SEQ ID NO:33), 5' GGGUUUU 3', 5' GGGGUUUUGGGG 3' (SEQ ID NO:34), GUUUUUG (SEQ ID NO 35), GGGGGGGUUGUGUGGGGG (SEQ ID NO:36), CCCCUUUUGGGGG (SEQ ID NO:37), GUUUGUGUGGGG (SEQ ID NO:38), GUUGUGUGGGGG (SEQ ID NO:39), UUUUUUGGGGG (SEQ ID NO:40), UUUUUGGGGG (SEQ ID NO:41), UUUUGGGGG (SEQ ID NO:19), or UUUUGGGG (SEQ ID NO:15).

The immunostimulatory ORN motif in some embodiments is a TLR8 motif. The TLR8 motif according to some aspects of the invention is N-U-R_1-R_2.

N is a ribonucleotide and N does not include a U. In some embodiments N is Adenosine or Cytosine (C) or derivatives thereof.

U is Uracil or a derivative thereof.

R is a ribonucleotide wherein at least one of R_1 and R_2 is Adenosine (A) or Cytosine or derivatives thereof. R is not U unless N-U-R_1-R_2 includes at least two A.

The ORN of the invention includes at least one and in some embodiments more than one (i.e., 2, 3, or 4) immunostimulatory motifs, N-U-R_1-R_2. The ORN including a TLR 8 motif may optionally also include a TLR7/8 motif.

N-U-R_1-R_2 may in some embodiments include at least 3 As or at least 2 Cs. Optionally, N-U-R_1-R_2 includes at least one G or C.

In other embodiments the TLR 8 motif is separated from a 5' ribonucleotide by a non-nucleotide linker. In yet other embodiments the TLR 8 motif is separated from a 3' ribonucleotide by a non-nucleotide linker. Optionally, the TLR 8 motif is separated from a 5' and 3' ribonucleotide by a non-nucleotide linker.

In other embodiments the TLR8 motif includes at least one AU. In yet other embodiments the TLR8 motif includes at least one CU.

In yet other embodiments the immunostimulatory ORN motif is a TLR7/8 motif. TLR7/8 motifs include, for instance, ribonucleotide sequences such as (i) 5'-C/U-U-G/U-U-3', (ii) 5'-R-U-R-G-Y-3', (iii) 5'-G-U-U-G-B-3', (iv) 5'-G-U-G-U-G/U-3', and (v) 5'-G/C-U-A/C-G-G-C-A-C-3'. C/U is cytosine (C) or uracil (U). G/U is guanine (G) or U. R is purine. Y is pyrimidine. B is U, G, or C. G/C is G or C. A/C is adenine (A) or C.

In various embodiments 5'-C/U-U-G/U-U-3' is CUGU, CUUU, UUGU, or UUUU.

In various embodiments 5'-R-U-R-G-Y-3' is GUAGU, GUAGC, GUGGU, GUGGC, AUAGU, AUAGC, AUGGU, or AUGGC. In one embodiment the base sequence is GUAGUGU.

In various embodiments 5'-G-U-U-G-B-3' is GUUGU, GUUGG, or GUUGC.

In various embodiments 5'-G-U-G-U-G/U-3' is GUGUG or GUGUU. In one embodiment the base sequence is GUGUUUAC.

In various embodiments 5'-G/C-U-A/C-G-G-C-A-C-3' is GUAGGCAC, GUCGGCAC, CUAGGCAC, or CUCGGCAC.

In some aspects the ORN of the invention is an ORN of 10-100 ribonucleotides in length comprising: $GG(R_1)_n\ (U)_{4\text{-}20}(R_2)_m$ GGGG (SEQ ID NO:29), $GG(R_1)_n\ (U)_{5\text{-}20}(R_2)_m$ GGGG (SEQ ID NO:30), or $GG(R_1)_n\ (U)_4(R_2)_m$ GGGG (SEQ ID NO:31).

R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker. U is Uridine or a derivative thereof. G is guanosine or a derivative

thereof. n=0-20 and m= 0-20. In some embodiments when $(R_1)_n$ is GG $(R_2)_m$ is not G or m is not = 0.

In other embodiments the ORN does not include specific modified phosphate linkages of formulas described herein (i), (ii), (iii) or any combinations thereof.

In some embodiments the ORN is

rG*rG*rG*rG*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:4),

rG*rG*rG*rG rU*rU*rG*rU*rU*rG*rU*rU*rG*rU rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:5), rG*rG*rG*rG*rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:6),

rG*rG*rG*rG-rU-rU-rU-rU-rU-rU-rU-rU-rU-rU-rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:8), rG*rU*rU*rG*rU*rG*rU*dG*dG*dG*dG*dG (SEQ ID NO:10), rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG*rG*rG (SEQ ID NO:23), rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG (SEQ ID NO:24), rG*rU*rU*rG*rU*rG*rU*rG*rG (SEQ ID NO:25), rU*rU*rG*rU*rG*rG*rG*rG*rG (SEQ ID NO:26), rU*rU*rU*rU*rG*rG*rG*rG*rG (SEQ ID NO:27), or rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG*rG (SEQ ID NO:21).

Optionally, the components of the formula are defined as one or more of the following: $(R_1)_n$ is GG, $(R_2)_m$ is GGG, $(U)_{4-20}$ is UUUUU, $(U)_{4-20}$ is UUUUUUU, $(U)_{4-20}$ is UUUUUUUUUU (SEQ ID NO:32), GG and $(R_1)_n$ are connected directly, GG and $(R_1)_n$ are connected via a 3'-3' linkage or GG and $(R_1)_n$ are connected by a spacer. In some embodiments the spacer is a non-nucleotide spacer such as a D-spacer or a linker.

The composition may further include a sterile carrier.

The ORN may be single stranded or double stranded or partially double-stranded. In some embodiments the ORN is not an siRNA or antisense oligonucleotide.

The ORN may include at least one phosphorothioate linkage. In some embodiments all internucleotide linkages of the ORN are phosphorothioate linkages. In other embodiments the ORN includes at least one phosphodiester-like linkage. Optionally, the phosphodiester-like linkage is a phosphodiester linkage.

In one aspect the invention provides an immunostimulatory composition including an immunostimulatory ORN of the invention and an adjuvant. In various embodiments the adjuvant is an adjuvant that creates a depot effect, an immune-

stimulating adjuvant, or an adjuvant that creates a depot effect and stimulates the immune system. In one embodiment the immunostimulatory composition according to this aspect of the invention is a conjugate of the immunostimulatory ORN and the adjuvant. In one embodiment according to this aspect of the invention the immunostimulatory ORN is covalently linked to the adjuvant. In other embodiments they are not conjugated.

The compositions of the invention can optionally include an antigen. Thus in one aspect the invention provides a vaccine, wherein the vaccine includes an immunostimulatory ORN of the invention and an antigen. In one aspect the invention provides a vaccine that includes a conjugate of an immunostimulatory ORN of the invention and an antigen. In one embodiment the conjugate according to this aspect of the invention includes the immunostimulatory ORN covalently linked to the antigen. In other embodiments they are not conjugated. In various embodiments the antigen can be an antigen *per se*. The antigen can be any antigen, including a cancer antigen, a microbial antigen, or an allergen.

In one aspect the invention provides an immunostimulatory composition including a conjugate of an immunostimulatory ORN of the invention and a lipophilic moiety. In one embodiment the immunostimulatory ORN is covalently linked to the lipophilic moiety. In one embodiment the lipophilic moiety is selected from the group consisting of cholesteryl, palmityl, and fatty acyl. In one embodiment the lipophilic moiety is a derivative of cholesterol, e.g., cholesteryl.

In one embodiment the immunostimulatory ORN includes at least one deoxyribonucleotide. The at least one deoxyribonucleotide generally can occur anywhere outside of the immunostimulatory RNA motif. In various embodiments the at least one deoxyribonucleotide is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 consecutive deoxyribonucleotides. Immunostimulatory ORN including nonconsecutive deoxyribonucleotides are also contemplated by the invention. In various embodiment the at least one deoxyribonucleotide is a 5′ end, a 3′ end, or both a 5′ end and a 3′ end of the immunostimulatory ORN. The at least one deoxyribonucleotide also corresponds to a DNA portion of a chimeric DNA:RNA molecule. In one embodiment a DNA component of the chimeric DNA:RNA molecule includes a CpG nucleic acid, i.e., a TLR9 agonist. In one embodiment the DNA and

RNA portions of the chimeric DNA:RNA molecule are covalently linked through an internucleotide phosphate bond. In another embodiment the DNA and RNA portions of the chimeric DNA:RNA molecule are covalently linked through a linker, e.g., a non-nucleotidic linker.

In one aspect the invention provides an immunostimulatory composition that includes a covalently closed, partially single-stranded, dumbbell-shaped nucleic acid molecule, wherein at least one single-stranded portion of the molecule includes an immunostimulatory RNA motif of the invention.

In one aspect the invention provides a pharmaceutical composition including the composition of any of the foregoing aspects of the invention, in association with a delivery vehicle such as a cationic lipid, a liposome, a cochleate, a virosome, an immune-stimulating complex (ISCOM), a microparticle, a microsphere, a nanosphere, a unilamellar vesicle (LUV), a multilamellar vesicle, an oil-in-water emulsion, a water-in-oil emulsion, an emulsome, and a polycationic peptide, and, optionally, a pharmaceutically acceptable carrier. In one embodiment according to this aspect of the invention the pharmaceutical composition includes an antigen. Further examples of delivery vehicles are described below.

The ORN may be formulated in a nebulizer or an inhaler, such as a metered dose inhaler or a powder inhaler. In some embodiments the ORN further includes an additional composition such as a chemotherapeutic agent, an anti-viral agent or a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be formulated for injection or mucosal administration.

Further according to these and other aspects of the invention, in various embodiments the immunostimulatory ORN can optionally include at least one 5'-5' internucleotide linkage, at least one 3'-3' internucleotide linkage, at least one 5'-5' internucleotide linkage that includes a linker moiety, at least one 3'-3' internucleotide linkage that includes a linker moiety, or any combination thereof. The linker moiety in one embodiment is a non-nucleotidic linker moiety.

Further still according to these and other aspects of the invention, in various embodiments the immunostimulatory ORN can optionally include at least one 2'-2' internucleotide linkage, at least one 2'-3' internucleotide linkage, at least 2'-5' internucleotide linkage, or any combination thereof. In a preferred embodiment the at

least one 2'-2' internucleotide linkage, at least one 2'-3' internucleotide linkage, or at least 2'-5' internucleotide linkage occurs outside of the immunostimulatory RNA motif.

Also according to these and other aspects of the invention, the immunostimulatory ORN in one embodiment includes at least one multiplier unit. Accordingly, in certain embodiments the immunostimulatory ORN of the invention can have a branched structure. Branched compositions can include 3′-5′, 5′-5′, 3′-3′, 2′-2′, 2′-3′, or 2′-5′ internucleotide linkages, in any combination. In one embodiment the immunostimulatory ORN includes at least two multiplier units, resulting in a so-called dendrimer. In addition, in certain embodiments the immunostimulatory ORN of the invention may include two or more immunostimulatory RNA motifs, arranged for example in tandem along a linear ORN, on different arms of a branched structure, or both in tandem along a linear ORN and on different arms of a branched structure. Branched structures, including dendrimers, can optionally include at least one immunostimulatory CpG nucleic acid, for example as a separate arm of a branched structure.

Further according to these and other aspects of the invention, in one embodiment the immunostimulatory ORN comprises at least one 2'-O-alkyl-modified, 2'-fluoro-arabino-modified G, or LNA-modified G.

Further according to these and other aspects of the invention, in one embodiment the immunostimulatory ORN does not include a CG DNA or RNA dinucleotide.

In another aspect the invention provides a method for modulating an immune response in a subject. The method according to this aspect of the invention includes the step of administering to a subject an effective amount of a composition of the invention. In some embodiments the ORN may be delivered to the subject to treat autoimmune disease or airway remodeling in the subject. The ORN may be administered with or without an antigen to the subject. Optionally the ORN is delivered by a route such as oral, nasal, sublingual, intravenous, subcutaneous, mucosal, respiratory, direct injection, and dermally. The ORN may be delivered to the subject in an effective amount to induce cytokine expression, such as IFNα.

In one aspect the invention provides a method of vaccinating a subject. The method according to this aspect of the invention includes the step of administering to the subject an antigen and an immunostimulatory ORN of the invention.

In one aspect the invention provides a method for treating a subject having or at risk of having an infectious disease. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of a composition of the invention. In one embodiment the method includes the step of administering to the subject an effective amount of an immunostimulatory ORN of the invention. In one embodiment the subject has a viral infection. The viral infection may be, for example, hepatitis B or hepatitis C. An anti-viral agent may be also administered to the subject. Optionally the anti-viral agent is linked to the ORN.

In one aspect the invention provides a method for treating a subject having or at risk of having a cancer. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of a composition of the invention. In one embodiment the method includes the step of administering to the subject an effective amount of an immunostimulatory ORN of the invention. In one embodiment a chemotherapeutic or radiation is also administered to the subject.

In one aspect the invention provides a method for treating a subject having or at risk of having an allergic condition. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of a composition of the invention. In one embodiment the method includes the step of administering to the subject an effective amount of an immunostimulatory ORN of the invention. In one embodiment the subject has allergic rhinitis.

In one aspect the invention provides a method for treating a subject having or at risk of having asthma. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of a composition of the invention. In one embodiment the method includes the step of administering to the subject an effective amount of an immunostimulatory ORN of the invention. In one embodiment the asthma is asthma exacerbated by viral infection. The ORN may be administered with or without an allergen.

In another aspect the invention provides a method for treating a subject having airway remodeling. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of an immunostimulatory ORN of the invention.

In one aspect the invention provides a method for increasing antibody-dependent cellular cytotoxicity (ADCC). The method according to this aspect of the invention includes the step of administering to a subject in need of increased ADCC an effective amount of an immunostimulatory ORN of the invention and an antibody to increase ADCC. In one embodiment the antibody is an antibody specific for a cancer antigen or other antigen expressed by a cancer cell. In one embodiment the antibody is an IgG antibody.

The invention in one aspect provides a method for enhancing epitope spreading. The method according to this aspect of the invention includes the sequential steps of contacting a cell of the immune system with an antigen and subsequently contacting the cell with at least two doses of an immunostimulatory ORN of the invention. In one embodiment the method is performed in vivo. The method in one embodiment includes the steps of administering to a subject a vaccine that includes an antigen and an adjuvant and subsequently administering to the subject at least two doses of an immunostimulatory ORN of the invention, in an effective amount to induce multiple epitope-specific immune responses. The method in one embodiment involves applying a therapeutic protocol which results in immune system antigen exposure in a subject, followed by administering at least two doses of an immunostimulatory ORN of the invention, in an effective amount to induce multiple epitope-specific immune responses. In various embodiments the therapeutic protocol is surgery, radiation, chemotherapy, other cancer medicaments, a vaccine, or a cancer vaccine. In one embodiment the at least two doses of the immunostimulatory ORN are administered at least one day to one week apart from one another. In one embodiment the at least two doses of the immunostimulatory ORN are administered at least one week to one month apart from one another. In one embodiment the at least two doses of the immunostimulatory ORN are administered at least one month to six months apart from one another.

In one aspect the invention is a method for stimulating production of IFN-α, by contacting a TLR7 expressing cell with an RNA oligonucleotide (ORN) of the invention in an effective amount to stimulate IFN-α production and wherein IFN-γ or IL-12 production in response to the ORN is not induced significantly relative to background. In some embodiments the TLR7 expressing cell is *in vitro* or *in vivo*. In one embodiment the ORN is an immunostimulatory ORN motif linked to a poly-G motif,

wherein the poly-G motif is 3' to the immunostimulatory ORN motif and the poly-G motif comprises at least 4 Gs. In other embodiments the ORN is $GG(R_1)_n (U)_{4-20}(R_2)_m$ GGGG wherein R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker, wherein n=0-20, wherein m= 0-20, U is Uridine or a derivative thereof, G is guanosine or a derivative thereof.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including," "comprising," or "having," "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

BRIEF DESCRIPTION OF DRAWINGS

The figures are illustrative only and are not required for enablement of the invention disclosed herein.

Figure 1 is two graphs showing cytokine production by human PBMC after contacting the cells with oligoribonucleotides (ORN). ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and supernatants were assayed 24 hours later for cytokine concentration by ELISA. Shown are TLR7/8 immunostimulatory ORN (SEQ ID NO:1 and 2) and three test sequences (SEQ ID NO:4, 5, and 8, see Table 1). The y-axes are IFN-α (Figure 1A) or IL-12p40 (Figure 1B) concentration in pg/ml and the x-axes are log ORN concentration in μM.

Figure 2 is two graphs showing cytokine production by human PBMC after contacting the cells with oligoribonucleotides (ORN). ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and supernatants were assayed 24 hours later for cytokine concentration by ELISA. Shown are TLR7/8 immunostimulatory ORN (SEQ ID NO:1 and 2) and three test sequences (SEQ ID NO:3,

6, and 7, see Table 1). The y-axes are IFN-α (Figure 2A) or IL-12p40 (Figure 2B) concentration in pg/ml and the x-axes are log ORN concentration in μM.

Figure 3 is two graphs showing cytokine production by human PBMC after contacting the cells with oligoribonucleotides (ORN). ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and supernatants were assayed 24 hours later for cytokine concentration by ELISA. Shown are TLR7/8 immunostimulatory ORN (SEQ ID NO:1) and four test sequences (SEQ ID NO:9-12). The y-axes are IFN-α (Figure 3A) or IFN-γ (Figure 3B) concentration in pg/ml and the x-axes are ORN concentration in μM.

Figure 4 is a graph showing IFN-α production by human PBMC after contacting the cells with oligoribonucleotides (ORN). ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and supernatants were assayed 24 hours later for cytokine concentration by ELISA Shown are SEQ ID NO:10 and the equivalent sequence with a modified G (7-deaza-rG), both with and without DOTAP (DO). The y-axis is IFN-α concentration in pg/ml and the x-axis is ORN concentration in μM.

Figure 5 is a graph showing IFN-α production by human PBMC after contacting the cells with oligoribonucleotides (ORN) with various 3' modifications. ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and supernatants were assayed 24 hours later for cytokine concentration by ELISA Shown are SEQ ID NO:12 and 10 test sequences. The y-axis is IFN-α concentration in pg/ml and the x-axis is ORN concentration in μM.

Figure is two graphs showing cytokine production by human PBMC after contacting the cells with oligoribonucleotides (ORN). ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and supernatants were assayed 24 hours later for cytokine concentration by ELISA. Shown are SEQ ID NO:21 and 5 test sequences, and the TLR7/8 immunostimulatory ORN SEQ ID NO:1.

Figure 7 is three graphs showing cytokine production by human PBMC after contacting the cells with oligoribonucleotides (ORN). demonstrates that ORN with poly rG stimulate TLR7-dependent IFN-α production in pDC that lack TLR8. Human PBMC (n=2) (Figure 7A), monocytes (Figure 7B) or pDC (Figure 7C) were stimulated with SEQ ID NO:14 (4μM), the immune stimulatory CpG ODN SEQ ID NO:28 (0.5μM), or

SEQ ID NO:12 (0.5μM) in the presence of DOTAP (20μg/ml) for 24 hours and IFN-α measured. The y-axis is the ORN, ODN, or medium tested and the x-axes are IFN-α concentration in pg/ml.

Figure 8 is four graphs showing stimulation of cytokine production in murine dentritic cells. Murine CD11+ splenocytes were harvested and treated with ORN for 20 hours. Supernatants were analyzed by ELISA for IFN-α (Figure 8A), IL-6 (Figure 8B), IL-12p40 (Figure 8C) and IP-10 (Figure 8D) concentration. Cells were treated with a know TLR 7/8 stimulatory ORN (SEQ ID NO:48) with DOTAP (DO), the small molecule R-848 which stimulates TLR7/8, the cholesterol tagged and 3'G stretch modified ORN of SEQ ID NO:12 (SEQ ID NO:11 and 12, respectively, both with and without DOTAP), and SEQ ID NO:12 with DOTAP. The y-axes are cytokine concentration in pg/ml and the x-axes are the treatment dose concentration in nM.

Figure 9 is four graphs showing stimulation of cytokine production *in vivo*. sv129 mice were injected intravenously with an unmodified ORN (SEQ ID NO:12), a cholesterol modified ORN of the same sequence (SEQ ID NO:11), an ORN with the same sequence and a 3' poly G stretch (SEQ ID NO:21), or R-848. All ORN were formulated with DOTAP at a 2:1 ratio (w/w). Mice were bled and serum analyzed for IFN-α (Figure 9A), IL-12p40 (Figure 9B), IP-10 (Figure 9C) and TNF-α (Figure 9D) concentration by ELISA. The y-axes are cytokine concentration in pg/ml and the x-axes are the treatment dose concentration in nM.

Figure 10 is two graphs showing stimulation of cytokine production *in vivo*. sv129 mice were injected intravenously. Serum was tested 3 hours after injection for cytokine production in mice after stimulation with ORN. Figure 10A shows stimulation with 30 μg SEQ ID NO:21 + DOTAP, 30 μg of SEQ ID NO:21 alone, or DOTAP or water alone. The x-axis is the dose given and the y-axis is IP-10 concentration in pg/ml. Figure 10B shows IP-10 concentration after injection with an unmodified ORN (SEQ ID NO:12), a cholesterol modified ORN of the same sequence (SEQ ID NO:11), or an ORN with the same sequence and a 3' poly G stretch (SEQ ID NO:21). The x-axis is the treatment dose in μg and the y-axis is IP-10 concentration in pg/ml.

Figure 11 is a graph showing IFN-α production by human PBMC after contacting the cells with oligoribonucleotides (ORN) with various 3' modifications. ORN (starting concentration: 2 μM + 50 μg/ml DOTAP) were incubated with human PBMC and

supernatants were assayed 24 hours later for cytokine concentration by ELISA Shown are the TLR7/8 stimulatory ORN SEQ ID NO:48 (with and without DOTAP) and 6 test sequences with modified 3' ends (no DOTAP). The y-axis is IFN-α concentration in pg/ml and the x-axis is ORN concentration in log μM.

DETAILED DESCRIPTION

Immunostimulatory oligoribonucleotides (ORN) have been described which appear to stimulate the human immune system in a TLR7 and/or TLR8 dependent manner. For instance, ORN containing GU rich and CU rich motifs lacking poly-G ends appear to act on TLR 7 and TLR8. ORN with AU rich motifs lacking poly-G ends appear to act on TLR8 only, as they stimulate cytokines e.g. TNF-α, IL-12 and IFN-γ associated with TLR9 activation, and not IFN-α, which is associated with TLR7 activation. For example, activation of monocytes is most likely a direct TLR8-mediated effect because monocytes are shown to express TLR8 but not TLR7, and secrete TNF-α upon ssRNA stimulation, whereas the IFFN-α producing pDC express TLR7 and no TLR8. Recently the inventors identified ORN with different immune profiles and defined motifs for the activation of RNA-mediated responses. Some of those ORN did not induce IFN-α production by human PBMC, but do induce significant amounts of TNF-α, IL-12 and IFN-γ, pointing to a stimulation of mainly TLR8 without significant stimulation of TLR7. These ORN are described at least in U.S. Patent Application Serial No.11/603,978.

The instant invention involves the unexpected finding that ORN having specific motifs can induce RNA-mediated pDC immune responses referred to as TLR7 mediated (such as IFN-α production) without inducing substantial amounts of TLR8-mediated (i.e. production of cytokines produced by TLR8 expressing cells such as TNF-α from monocytes) immune activation. As used herein, a "substantial amount" shall mean that the levels induced are minimal when compared with levels induced by ORN such as those containing GU rich and CU rich motifs lacking poly-G ends mentioned above, or other ORN that appear to stimulate TLR8. Thus, the ORN of the instant invention induce less of the cytokines typical for an RNA TLR8 or 7/8 ligand, e.g., pro-inflammatory cytokines TNF-α, IL-6.

The class of ORN described herein includes an immunostimulatory ORN motif directly or indirectly flanked by a 3' poly G motif and optionally a 5' poly-G motif and is associated with an immune profile that is characteristic for the almost exclusive activation of a TLR7 like immune response. For example, as shown in Figure 1, ORN of the invention SEQ ID NO:4, SEQ ID NO:8 and SEQ ID NO:5 induce very high amounts of IFN-α when formulated with DOTAP with no significant induction of other responses like IFN-γ or IL-12. In contrast, the positive control ORN known to stimulate both TLR7 and TLR8 associated cytokines, SEQ ID NO:1 and 2, induced both high amounts of IFN-α and high amounts of IL-12p40. Quite surprisingly it was also discovered according to the invention that immunostimulatory ORN motifs such as those which mediate TLR7/8 and TLR8 responses produce a TLR7 immune profile when one or more poly-G motifs is incorporated into the ORN.

The immunostimulatory RNA motif according to some aspects of the invention is an immunostimulatory ORN motif linked to a poly-G motif, wherein the poly-G motif is 3' to the immunostimulatory ORN motif and the poly-G motif comprises at least 4 Gs.

In some but not all embodiments the ORN specifically includes TLR7/8 and/or TLR8 motifs. A TLR7/8 motif may include for example a ribonucleotide sequence such as 5'-C/U-U-G/U-U-3', 5'-R-U-R-G-Y-3', 5'-G-U-U-G-B-3', 5'-G-U-G-U-G/U-3', or 5'-G/C-U-A/C-G-G-C-A-C-3'. C/U is cytosine (C) or uracil (U), G/U is guanine (G) or U, R is purine, Y is pyrimidine, B is U, G, or C, G/C is G or C, and A/C is adenine (A) or C. The 5'-C/U-U-G/U-U-3' may be CUGU, CUUU, UUGU, or UUUU. In various embodiments 5'-R-U-R-G-Y-3' is GUAGU, GUAGC, GUGGU, GUGGC, AUAGU, AUAGC, AUGGU, or AUGGC. In one embodiment the base sequence is GUAGUGU. In various embodiments 5'-G-U-U-G-B-3' is GUUGU, GUUGG, or GUUGC. In various embodiments 5'-G-U-G-U-G/U-3' is GUGUG or GUGUU. In one embodiment the base sequence is GUGUUUAC. In various other embodiments 5'-G/C-U-A/C-G-G-C-A-C-3' is GUAGGCAC, GUCGGCAC, CUAGGCAC, or CUCGGCAC.

A TLR8 motif is, for instance, N-U-R_1-R_2, wherein N is a ribonucleotide and N does not include a U, U is Uracil or a derivative thereof and wherein R is a ribonucleotide wherein at least one of R_1 and R_2 is Adenosine (A) or Cytosine or derivatives thereof. R is not U unless N-U-R_1-R_2 includes at least two A. In some

embodiments, N is Adenosine or Cytosine (C) or derivatives thereof. Optionally the ORN includes more that one N-U-R_1-R_2 motif.

In other embodiments the ORN specifically excludes TLR7/8 and/or TLR8 motifs.

The ORN may have the following structure: $GG(R_1)_n\ (U)_{4\text{-}20}(R_2)_m GGGG$ (SEQ ID NO:29). In other aspects the RNA motif is $GG(R_1)_n\ (U)_{5\text{-}20}(R_2)_m$ GGGG (SEQ ID NO:30). In other aspects the RNA motif is $GG(R_1)_n\ (U)_4(R_2)_m$, GGGG (SEQ ID NO:31) wherein when $(R_1)_n$ is GG $(R_2)_m$ is not G or m is not = 0.

Poly U refers to a stretch of at least 4 Us. Poly G refers to a stretch of at least 2 Gs.

R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker. In some embodiments $(R_1)_n$ is GG. In other embodiments $(R_2)_m$ is GGG.

U is Uridine or a derivative thereof. $(U)_{4\text{-}20}$ or $(U)_{5\text{-}20}$ may be UUUUU, UUUUUUU, or UUUUUUUUUU (SEQ ID NO:32) for instance.

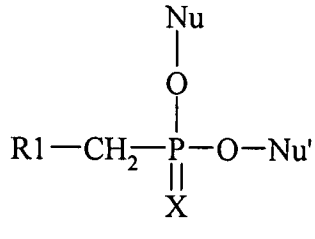
G is guanosine or a derivative thereof. n=0-20. m= 0-20.

In some embodiments the RNA motif is an ORN of any one of SEQ ID NOs. 4-6 and 8-12.

In some embodiments the ORN is not 5' GGGGUUUUGGGGG 3' (SEQ ID NO:33), 5' GGGGUUUUGGGG 3' (SEQ ID NO:34), GUUUUUG (SEQ ID NO 35), GGGGGGGUUGUGUGGGGG (SEQ ID NO:36), CCCCUUUUGGGGG (SEQ ID NO:37), GUUUGUGUGGGG (SEQ ID NO:38), GUUGUGUGGGGG (SEQ ID NO:39), UUUUUUGGGGG (SEQ ID NO:40), UUUUUGGGGG (SEQ ID NO:41), UUUUGGGGG (SEQ ID NO:19), or UUUUGGGG (SEQ ID NO:15).

In other embodiments the ORN does not include a modified phosphate linkage selected from the group consisting of:

(i)



Formula I

wherein

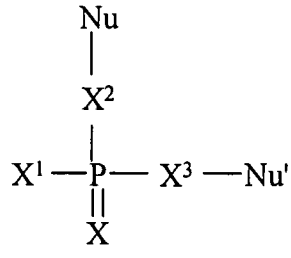
R1 is hydrogen (H), COOR, OH, C1-C18 alkyl, C_6H_5, or $(CH_2)_m$-NH-R2, wherein R is H or methyl, butyl, methoxyethyl, pivaloyl oxymethyl, pivaloyl oxybenzyl, or S-pivaloyl thioethyl; R2 is H, C1-C18 alkyl, or C2-C18 acyl; and m is 1 to 17;

X is oxygen (O) or sulfur (S); and

each of Nu and Nu' independently is a nucleoside or nucleoside analog;

with the proviso that if R1 is H, then X is S;

(ii)



Formula II

wherein

X is O or S;

X^1 is OH, SH, BH_3, OR3, or NHR3, wherein R3 is C1-C18 alkyl;

each of X^2 and X^3 independently is O, S, CH_2, or CF_2; and

each of Nu and Nu' independently is a nucleoside or nucleoside analog;

with the proviso that

(a) at least one of X, X^2, and X^3 is not O or X^1 is not OH,

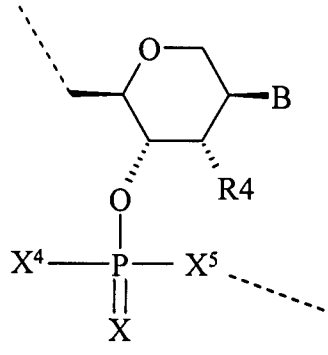
(b) if X^1 is SH, then at least one of X, X^2, and X^3 is not O,

(c) if X and X^2 are O and if X^1 is OH, then X^3 is not S and Nu is 3'Nu and Nu' is 5'Nu', and

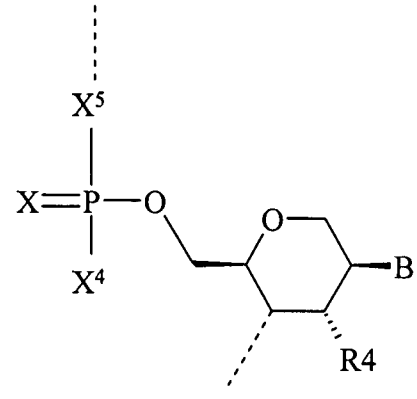
(d) if X^1 is BH_3, then at least one of X, X^2, or X^3 is S; and

(iii) any combination of (i) and (ii)

or at least one nucleotide analog provided as Formula IIIA or Formula IIIB



Formula IIIA



Formula IIIB

wherein

R4 is H or OR, wherein R is H or C1-C18 acyl;

B is a nucleobase, a modified nucleobase, or H;

each of X and X^5 independently is O or S; and

X^4 is OH, SH, methyl, or NHR5, wherein R5 is C1-C18 alkyl; and

each dashed line independently represents an optional bond to an adjacent unit, hydrogen, or an organic radical;

with the proviso that at least one of X and X^5 is not O or X^4 is not OH.

The ORN may be single or double stranded. According to the methods of the invention, the modified ORN are not designed to comprise a sequence complementary to that of a coding sequence in a human cell, and are therefore not considered to be antisense ORN or silencing RNA (siRNA). An ORN which is "not complementary" is one that does not comprise a sequence capable of hybridizing strongly with one particular coding region in the target cell. Therefore, administration of an ORN which is not complementary as used herein will not result in gene silencing, especially as the ORN described in this invention are single-stranded compared to the double-stranded molecules used as silencing RNAs.

In some embodiments the ORN of the invention is between 10 and 30 nucleotides in length. In some embodiments the ORN is between 10 and 50 nucleotides in length. In some embodiments the ORN of the invention is between 10 and 100 nucleotides in length.

In some embodiments the ORN have a backbone that may be stabilized. In one embodiment the backbone is a sugar phosphate backbone that includes at least one phosphorothioate internucleotide linkage. In one embodiment the backbone is completely phosphorothioate. The ORN may include at least one phosphodiester-like linkage. In some instances the phosphodiester-like linkage is a phosphodiester linkage.

Clear differences between production of IFN-α and other pro-inflammatory cytokines such as IFN-γ and IL-12 were observed for ORN of the invention and ORN having a TLR7/8 motif, i.e. GU-containing repetitions or a LTR8 motif, i.e. AU-containing repetitions. The ORN of the invention having a poly G-poly U-poly G motif, for example SEQ ID NOs 4-6 and 8-12 revealed IFN-α cytokine production upon PBMC and pDC stimulation but not IFN-γ and IL-12.

Thus, the ORN of the invention have the ability to induce an immune response inducing significant amounts of IFN-α or IFN-α related molecules relative to background. A significant amount of IFN-α or IFN-α related molecules relative to background is preferably more than 20% change in levels of IFN-α or IFN-α related molecules relative to background. In some embodiments it is more than 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%. In other embodiments the amount of IFN-α induced by the ORN of the invention is more than or equal to 20% of the IFN-α induced by a TLR7/8 ORN or a TLR 8 ORN. The amount of IFN-alpha induced by the ORN of the invention may optionally be more than 300pg/ml in an *in vitro* assay or may have an EC50 of greater than 1.5μM.

An IFN-α related molecule, as used herein, is a cytokine or factor that is related to the expression of IFN-α. These molecules include but are not limited to MIP1-β, IP-10 and MIP1-α.

The invention relates generally to immunostimulatory oligoribonucleotides that include one or more immunostimulatory RNA motifs, immunostimulatory compositions containing one or more immunostimulatory ORN of the invention, and methods for use of the immunostimulatory ORN and immunostimulatory compositions of the invention.

As used herein, the terms "RNA" shall refer to two or more ribonucleotides (i.e., molecules each comprising a ribose sugar linked to a phosphate group and to a purine or pyrimidine nucleobase (e.g., guanine, adenine, cytosine, or uracil)) covalently linked together by 3′-5′ phosphodiester linkage(s).

In different embodiments the immunostimulatory ORN including the immunostimulatory RNA motif can include a single motif or more than one immunostimulatory RNA motif. It is believed that there may be an advantage to having two or more immunostimulatory RNA motifs in a single immunostimulatory ORN, for example if the motifs are spaced such that the immunostimulatory ORN can engage two or more TLRs. For example, the immunostimulatory ORN could engage two or more TLR7 receptors thereby amplifying or modifying the resulting immunostimulatory effect.

When there is more than one immunostimulatory RNA motif in the immunostimulatory ORN, the motifs generally can occur at any position along the immunostimulatory ORN. For example, when there are two motifs, they may each occur at an end of the immunostimulatory ORN. Alternatively, one motif can occur at an end and one motif can be flanked on both of its ends by at least one additional nucleotide of the immunostimulatory ORN. In yet another embodiment each motif can be flanked on both of its ends by at least one additional nucleotide of the immunostimulatory ORN.

Immunostimulatory ORN include but are not limited to the following, shown 5′ to 3′ reading left to right:

rG*rG*rG*rG rU*rU*rU*rU*rU*rU*rU*rU*rU*rU rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:4),

rG*rG*rG*rG rU*rU*rG*rU*rU*rG*rU*rU*rG*rU rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:5),

rG*rG*rG*rG rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:6),

rG*rG*rG*rG-rU-rU-rU-rU-rU-rU-rU-rU-rU-rU- rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:8),

rG*rU*rU*rG*rU*rG*rU*dG*dG*dG*dG*dG (SEQ ID NO:10),

rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG*rG (SEQ ID NO:21),

rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*G*G*G*G*G (SEQ ID NO:42).

Other immunostimulatory ORN sequences as well as control sequences are found in Table 1, below.

As mentioned above, RNA is a polymer of ribonucleotides joined through 3′-5′ phosphodiester linkages. In certain embodiments the immunostimulatory ORN of the invention are RNA. However, the immunostimulatory ORN of the invention are not limited to RNA, as will be described below.

An immunostimulatory ORN of the invention can in one embodiment include one or more modified nucleobases i.e., derivatives of A, C, G, and U. Specific embodiments of these modified nucleobases include but are not limited to 5-substituted cytosines (e.g. 5-methyl-cytosine, 5-fluoro-cytosine, 5-chloro-cytosine, 5-bromo-cytosine, 5-iodo-cytosine, 5-hydroxy-cytosine, 5-hydroxymethyl-cytosine, 5-difluoromethyl-cytosine, and unsubstituted or substituted 5-alkynyl-cytosine), 6-substituted cytosines, N4-substituted cytosines (e.g. N4-ethyl-cytosine), 5-aza-cytosine, 2-mercapto-cytosine, isocytosine, pseudo-isocytosine, cytosine analogs with condensed ring systems (e.g. N,N'-propylene cytosine or phenoxazine), and uracil and its derivatives (e.g. 5-fluoro-uracil, 5-bromo-uracil, 5-bromovinyl-uracil, 4-thio-uracil, 5-hydroxy-uracil, 5-propynyl-uracil), thymine derivatives (e.g. 2-thiothymine, 4-thiothymine, 6-substituted thymines), guanosine derivatives (7-deazaguanine, 7-deaza-7-substituted guanine (such as 7-deaza-7-(C2-C6)alkynylguanine), 7-deaza-8-substituted guanine, hypoxanthine, N2-substituted guanines (e.g. N2-methyl-guanine), 8-substituted guanine (e.g. 8-hydroxyguanine and 8-bromoguanine), and 6-thioguanine), or adenosine derivatives (5-amino-3-methyl-3H,6H-thiazolo[4,5-d]pyrimidine-2,7-dione, 2,6-diaminopurine, 2-aminopurine, purine, indole, adenine, substituted adenines (e.g. N6-methyl-adenine, 8-oxo-adenine)). The base can also be substituted by a universal base (e.g. 4-methyl-indole, 5-nitro-indole, 3-nitropyrrole, P-base, and K-base), an aromatic ring system (e.g. benzimidazole or dichloro- benzimidazole, 1-methyl-1H-[1,2,4]triazole-3-carboxylic acid amide) an aromatic ring system (e.g. fluorobenzene or difluorobenzene) or a hydrogen atom (dSpacer). Preferred base modifications are uracil and 7-deaza-guanine. These modified U nucleobases and their corresponding ribonucleosides are available from commercial suppliers.

Specific embodiments of modified G nucleobases include N^2-dimethylguanine, 7-deazaguanine, 8-azaguanine, 7-deaza-7-substituted guanine, 7-deaza-7-(C2-C6)alkynylguanine, 7-deaza-8-substituted guanine, 8-hydroxyguanine, and 6-thioguanine. In one embodiment the modified G nucleobase is 8-hydroxyguanine. These modified G nucleobases and their corresponding ribonucleosides are available from commercial suppliers.

In certain embodiments at least one β-ribose unit may be replaced by β-D-deoxyribose or a modified sugar unit, wherein the modified sugar unit is for example

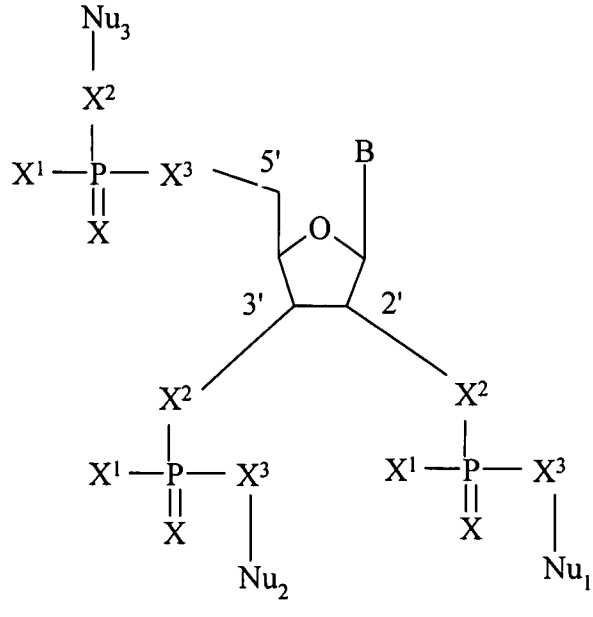
selected from β-D-ribose, α-D-ribose, β-L-ribose (as in 'Spiegelmers'), α-L-ribose, 2'-amino-2'-deoxyribose, 2'-fluoro-2'-deoxyribose, 2'-O-(C1-C6)alkyl-ribose, preferably 2'-O-(C1-C6)alkyl-ribose is 2'-O-methylribose, 2'-O-(C2-C6)alkenyl-ribose, 2'-[O-(C1-C6)alkyl-O-(C1-C6)alkyl]-ribose, LNA and α-LNA (Nielsen P et al. (2002) *Chemistry-A European Journal* 8:712-22), β-D-xylo-furanose, α-arabinofuranose, 2'-fluoro arabinofuranose, and carbocyclic and/or open-chain sugar analogs (described, for example, in Vandendriessche et al. (1993) *Tetrahedron* 49:7223) and/or bicyclosugar analogs (described, for example, in Tarkov M et al. (1993) *Helv Chim Acta* 76:481).

Individual ribonucleotides and ribonucleosides of the immunostimulatory ORN of the invention may alternatively be linked by non-nucleotidic linkers, in particular abasic linkers (dSpacers), triethylene glycol units, or hexaethylene glycol units. Additional linkers are alkylamino linkers, such as C3, C6, and C12 aminolinkers, and also alkylthiol linkers, such as C3 or C6 thiol linkers. Individual nucleotides and ribonucleosides of the immunostimulatory ORN of the invention may alternatively be linked by aromatic residues which may be further substituted by alkyl or substituted alkyl groups.

RNA is a polymer of ribonucleotides joined through 3′-5′ phosphodiester linkages. Nucleotides of the immunostimulatory ORN of the invention can also be joined through 3′-5′ phosphodiester linkages. However, the invention also encompasses immunostimulatory ORN having unusual internucleotide linkages, including specifically 5′-5′, 3′-3′, 2′-2′, 2′-3′, and 2′-5′ internucleotide linkages. In one embodiment such unusual linkages are excluded from the immunostimulatory RNA motif, even though one or more of such linkages may occur elsewhere within the immunostimulatory ORN. For immunostimulatory ORN having free ends, inclusion of one 3′-3′ internucleotide linkage can result in an immunostimulatory ORN having two free 5′ ends. Conversely, for immunostimulatory ORN having free ends, inclusion of one 5′-5′ internucleotide linkage can result in an immunostimulatory ORN having two free 3′ ends.

An immunostimulatory composition of this invention can contain two or more immunostimulatory RNA motifs which can be linked through a branching unit. The internucleotide linkages can be 3′-5′, 5′-5′, 3′-3′, 2′-2′, 2′-3′, or 2′-5′ linkages. Thereby, the nomenclature 2′-5′ is chosen according to the carbon atom of ribose. The unusual internucleotide linkage can be a phosphodiester linkage, but it can alternatively be

modified as phosphorothioate or any other modified linkage as described herein. The formula below shows a general structure for branched immunostimulatory ORN of the invention via a nucleotidic branching unit. Thereby Nu_1, Nu_2, and Nu_3 can be linked through 3'-5', 5'-5', 3'-3', 2'-2', 2'-3', or 2'-5' -linkages. Branching of immunostimulatory ORN can also involve the use of non-nucleotidic linkers and abasic spacers. In one embodiment, Nu_1, Nu_2, and Nu_3 represent identical or different immunostimulatory RNA motifs. In another embodiment, Nu_1, Nu_2, and Nu_3 comprise at least one immunostimulatory RNA motif and at least one immunostimulatory CpG DNA motif.



The immunostimulatory ORN may contain a doubler or trebler unit (Glen Research, Sterling, VA), in particular those immunostimulatory ORN with a 3'-3' linkage. A doubler unit in one embodiment can be based on 1,3-bis-[5-(4,4'-dimethoxytrityloxy)pentylamido]propyl-2-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. A trebler unit in one embodiment can be based on incorporation of Tris-2,2,2-[3-(4,4'-dimethoxytrityloxy)propyloxymethyl]ethyl-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Branching of the immunostimulatory ORN by multiple doubler, trebler, or other multiplier units leads to dendrimers which are a further embodiment of this invention. Branched immunostimulatory ORN may lead to crosslinking of receptors for immunostimulatory RNA such as TLR3, TLR7, and TLR8, with distinct immune effects compared to non-branched forms of the immunostimulatory ORN. In addition, the synthesis of branched or otherwise multimeric

immunostimulatory ORN may stabilize RNA against degradation and may enable weak or partially effective RNA sequences to exert a therapeutically useful level of immune activity. The immunostimulatory ORN may also contain linker units resulting from peptide modifying reagents or oligonucleotide modifying reagents (Glen Research). Furthermore, the immunostimulatory ORN may contain one or more natural or unnatural amino acid residues which are connected to the polymer by peptide (amide) linkages.

The compositions of the invention encompass polymers with and without secondary or higher order structure. For example, the polymer in one embodiment includes a sequence of nucleosides, nucleoside analogs, or a combination of nucleosides and nucleoside analogs capable of forming secondary structure provided by at least two adjacent hydrogen-bonded base pairs. In one embodiment the at least two adjacent hydrogen-bonded base pairs involve two sets of at least 3 consecutive bases. The consecutive nature of involved bases is thermodynamically advantageous for forming a so-called clamp. However, consecutive bases may not be required, particularly where there is high GC content and/or extended sequence. Typically there will be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 base pairs. A hydrogen-bonded base pair in one embodiment can be classical Watson-Crick base pair, i.e., G-C, A-U, or A-T. In other embodiments a hydrogen-bonded base pair can be a non-classical base pair, such as G-U, G-G, G-A, or U-U. In yet other embodiments a hydrogen-bonded base pair can be a Hoogsteen or other base pair.

In one embodiment the secondary structure is a stem-loop secondary structure. A stem-loop or hairpin secondary structure can arise through intramolecular hydrogen-bonded base pairing between complementary or at least partially complementary sequences. The complementary or at least partially complementary sequences represent perfect or interrupted inverted repeat sequences, respectively. For example, a polymer having a base sequence provided by 5′-X_1-X_2-X_3...X_3′-X_2′-X_1′-3′, wherein each of X_1 and X_1′, X_2 and X_2′, and X_3 and X_3′ can form a hydrogen-bonded base pair, may include a perfect or interrupted inverted repeat and has the potential to fold on itself and form a stem-loop secondary structure. It will be appreciated that a polymer having a base sequence provided by 5′-X_1-X_2-X_3...X_3′-X_2′-X_1′-3′, wherein each of X_1 and X_1′, X_2 and X_2′, and X_3 and X_3′ can form a hydrogen-bonded base pair, also has the potential to form intermolecular complexes through intermolecular hydrogen-bonded base pairs. Where

there are two or more inverted repeats, individual polymers can also interact to form not only dimeric intermolecular complexes but also higher-order intermolecular complexes or structures. Persons skilled in the art will recognize that conditions and/or sequences can be selected so as to favor formation of one type of secondary structure over another.

The 3′-5′, 5′-5′, 3′-3′, 2′-2′, 2′-3′, and 2′-5′ internucleotide linkages can be direct or indirect. Additionally the poly U motif may be directly or indirectly linked to the poly G motifs. Further, any of the nucleotides of the ORN formula may be linked directly or indirectly to one another. For instance GG and $(R_1)_n$ may be connected directly. The direct linkage may be a 3'-3' linkage. Alternatively GG and $(R_1)_n$ may be linked indirectly i.e., connected by a spacer or non-nucleotidic linker.

"Direct linkages" in this context refers to a phosphate or modified phosphate linkage as disclosed herein, without an intervening linker moiety. An intervening linker moiety is an organic moiety distinct from a phosphate or modified phosphate linkage as disclosed herein, which can include, for example, polyethylene glycol, triethylene glycol, hexaethylene glycol, dSpacer (i.e., an abasic deoxynucleotide), doubler unit, or trebler unit. Indirect linkages include nucleotidic and non-nucleotidic linkers and spacers.

In certain embodiments the immunostimulatory ORN is conjugated to another entity to provide a conjugate. For example, the immunostimulatory ORN can be conjugated to a peptide, protein, small molecular weight ligand, lipid moiety, or nucleic acid. As used herein a conjugate refers to a combination of any two or more entities bound to one another by any physicochemical means, including hydrophobic interaction and covalent coupling.

In another embodiment, the immunostimulatory ORN may be conjugated to a small molecular weight ligand which is recognized by an immunomodulatory receptor. This receptor is preferably a member of the TLR family, such as TLR2, TLR3, TLR4, TLR7, TLR8, or TLR9. The small molecular weight ligands are mimics of the natural ligands for these receptors. Examples include but are not limited to R-848 (Resiquimod), R-837 (Imiquimod; ALDARA™, 3M Pharmaceuticals), 7-deaza-guanosine, 7-thia-8-oxo-guansosine, and 7-allyl-8-oxo-guansosine (Loxoribine) which stimulate either TLR7 or TLR8. D-Glucopyranose derivatives, such as 3D-MPL (TLR4 ligand), may also be conjugated to the immunostimulatory ORN. Pam3-Cys is an example of a TLR2 ligand which can be conjugated to immunostimulatory ORN. Oligodeoxynucleotides

containing CpG motifs are TLR9 ligands, and these can also be conjugated to immunostimulatory ORN of the invention. In one embodiment, at least one oligodeoxynucleotide comprising a CpG motif effective for stimulating TLR9 signaling is conjugated to an immunostimulatory ORN of the invention. Conjugation of ligands for different TLRs into one molecule may lead to multimerisation of receptors which results in enhanced immune stimulation or a different immunostimulatory profile from that resulting from any single such ligand. In other embodiments CpG ODN are admixed without being conjugated or co-administered in therapeutic methods.

In one aspect the invention provides a conjugate of an immunostimulatory ORN of the invention and a lipophilic moiety. In certain embodiments the immunostimulatory ORN is covalently linked to a lipophilic moiety. The lipophilic moiety generally will occur at one or more ends of an immunostimulatory ORN having free ends, although in certain embodiments the lipophilic moiety can occur elsewhere along the immunostimulatory ORN and thus does not require the immunostimulatory ORN have a free end. In one embodiment the immunostimulatory ORN has a 3′ end and the lipophilic moiety is covalently linked to the 3′ end. The lipophilic group in general can be a cholesteryl, a modified cholesteryl, a cholesterol derivative, a reduced cholesterol, a substituted cholesterol, cholestan, C16 alkyl chain, a bile acid, cholic acid, taurocholic acid, deoxycholate, oleyl litocholic acid, oleoyl cholenic acid, a glycolipid, a phospholipid, a sphingolipid, an isoprenoid, such as steroids, vitamins, such as vitamin E, saturated fatty acids, unsaturated fatty acids, fatty acid esters, such as triglycerides, pyrenes, porphyrines, Texaphyrine, adamantane, acridines, biotin, coumarin, fluorescein, rhodamine, Texas-Red, digoxygenin, dimethoxytrityl, t-butyldimethylsilyl, t-butyldiphenylsilyl, cyanine dyes (e.g. Cy3 or Cy5), Hoechst 33258 dye, psoralen, or ibuprofen. In certain embodiments the lipophilic moiety is chosen from cholesteryl, palmityl, and fatty acyl. In one embodiment the lipohilic moiety is cholesteryl. It is believed that inclusion of one or more of such lipophilic moieties in the immunostimulatory ORN of the invention confers upon them yet additional stability against degradation by nucleases. Where there are two or more lipophilic moieties in a single immunostimulatory ORN of the invention, each lipophilic moiety can be selected independently of any other.

In one embodiment the lipophilic group is attached to a 2'-position of a nucleotide of the immunostimulatory ORN. A lipophilic group can alternatively or in addition be linked to the heterocyclic nucleobase of a nucleotide of the immunostimulatory ORN. The lipophilic moiety can be covalently linked to the immunostimulatory ORN via any suitable direct or indirect linkage. In one embodiment the linkage is direct and is an ester or an amide. In one embodiment the linkage is indirect and includes a spacer moiety, for example one or more abasic nucleotide residues, oligoethyleneglycol, such as triethyleneglycol (spacer 9) or hexaethylenegylcol (spacer 18), or an alkane-diol, such as butanediol.

In one embodiment the immunostimulatory ORN of the invention is combined with a cationic lipid. Cationic lipids are believed to assist in trafficking of the immunostimulatory ORN into the endosomal compartment, where TLR7 is found. In one embodiment the cationic lipid is DOTAP (N-[1-(2,3-dioleoyloxy)propy- l]-N,N,N-trimethylammonium methyl-sulfate). DOTAP is believed to transport polymers into cells and specifically traffic to the endosomal compartment, where it can release the polymer in a pH-dependent fashion. Once in the endosomal compartment, the polymers can interact with certain intracellular TLRs, triggering TLR-mediated signal transduction pathways involved in generating an immune response. Other agents with similar properties including trafficking to the endosomal compartment can be used in place of or in addition to DOTAP. Other lipid formulations include, for example, EFFECTENE® (a non-liposomal lipid with a special DNA condensing enhancer) and SUPERFECT® (a novel acting dendrimeric technology), SMARTICLES® (charge reversible particles that become positively charged when they cross cell membranes) and Stable Nucleic Acid Lipid Particles (SNALPs) which employ a lipid bilayer. Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN® and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis G (1985) *Trends Biotechnol* 3:235-241. In other embodiments the immunostimulatory polymers of the invention are combined with microparticles, cyclodextrins, nanoparticles, niosomes, dendrimers, polycytionic peptides, virosomes and virus-like

particles, or ISCOMS®. In other embodiments the ORN is free of a cationic lipid carrier. One advantage of the ORN of the invention is that they do not require such carriers.

In one embodiment the immunostimulatory ORN of the invention are in the form of covalently closed, dumbbell-shaped molecules with both primary and secondary structure. As described below, in one embodiment such cyclic oligoribonucleotides include two single-stranded loops connected by an intervening double-stranded segment. In one embodiment at least one single-stranded loop includes an immunostimulatory RNA motif of the invention. Other covalently closed, dumbbell-shaped molecules of the invention include chimeric DNA:RNA molecules in which, for example, the double-stranded segment is at least partially DNA (e.g., either homodimeric dsDNA or heterodimeric DNA:RNA) and at least one single-stranded loop includes an immunostimulatory RNA motif of the invention. Alternatively, the double stranded segment of the chimeric molecule is RNA.

In certain embodiments the immunostimulatory ORN is isolated. An isolated molecule is a molecule that is substantially pure and is free of other substances with which it is ordinarily found in nature or in *in vivo* systems to an extent practical and appropriate for its intended use. In particular, the immunostimulatory ORN are sufficiently pure and are sufficiently free from other biological constituents of cells so as to be useful in, for example, producing pharmaceutical preparations. Because an isolated immunostimulatory ORN of the invention may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the immunostimulatory ORN may comprise only a small percentage by weight of the preparation. The immunostimulatory ORN is nonetheless substantially pure in that it has been substantially separated from the substances with which it may be associated in living systems.

For use in the instant invention the immunostimulatory ORN of the invention can be synthesized de novo using or adapted from any of a number of procedures well known in the art. For example, the β-cyanoethyl phosphoramidite method (Beaucage SL et al. (1981) *Tetrahedron Lett* 22:1859); nucleoside H-phosphonate method (Garegg P et al. (1986) *Tetrahedron Lett* 27:4051-4; Froehler BC et al. (1986) *Nucl Acid Res* 14:5399-407; Garegg P et al. (1986) *Tetrahedron Lett* 27:4055-8; Gaffney BL et al. (1988) *Tetrahedron Lett* 29:2619-22). These chemistries can be performed by a variety

of automated nucleic acid synthesizers available in the market. Additional synthesis methods useful according to the instant invention are disclosed in Uhlmann E et al. (1990) *Chem Rev* 90:544-84, and Goodchild J (1990) *Bioconjugate Chem* 1:165.

Oligoribonucleotide synthesis can be performed either in solution or on a solid-phase support. In solution, block coupling reactions (dimers, trimers, tetramers, etc.) are preferred, while solid-phase synthesis is preferably performed in a stepwise process using monomeric building blocks. Different chemistries, such as the phosphotriester method, H-phosphonate method, and phosphoramidite method, have been described (Eckstein F (1991) *Oligonucleotides and Analogues, A Practical Approach*, IRL Press, Oxford). While in the phosphotriester method the reactive phosphorus group is in the oxidation state +V, the more reactive Phosphor +III derivatives are used in the coupling reactions according to the phosphoramidite and H-phosphonate approaches. In the latter two approaches, phosphorus is oxidized after the coupling step to yield the stable P(V) derivatives. If the oxidizer is iodine/water/base, then phosphodiesters are obtained after deprotection. In contrast, if the oxidizer is a sulfurizing agent, such as Beaucage's Reagent, then phosphorothioates are obtained after deprotection.

An efficient method for oligoribonucleotide synthesis is the combination of solid-support synthesis using phosphoramidite chemistry as originally described for oligodeoxynucleotides by Matteucci and Caruthers. Matteucci MD et al. (1981) *J Am Chem Soc* 103:3185.

Synthesis of oligoribonucleotides is similar to oligodeoxynucleotides, with the difference that the 2′-hydroxy group present in oligoribonucleotides must be protected by a suitable hydroxy protecting group. The monomers can be protected e.g. by 2′-O-t-butyldimethylsilyl (TBDMS) group in the RNA monomeric building blocks. However, RNA synthesis using monomers containing the 2′-O-TriisopropylsilylOxyMethyl (TOM) group (TOM-Protecting-Group™) has been reported to yield higher coupling efficiency, because the TOM-Protecting-Group exhibits lower steric hindrance than the TBDMS group. While the TBDMS protecting group is removed using fluoride, fast deprotection is achieved for the TOM group using methylamine in ethanol/water at room temperature. In oligo(ribo)nucleotide synthesis, chain elongation from 3′- to 5′-end is preferred, which is achieved by coupling of a ribonucleotide unit having a 3′-phosphor (III) group or its activated derivative to a free 5′-hydroxy group of another nucleotide unit.

Synthesis can be conveniently performed using an automated DNA/RNA synthesizer. Thereby, synthesis cycles as recommended by the suppliers of the synthesizers can be used. For ribonucleoside phosphoramidite monomers, coupling times are longer (e.g., 400 sec) as compared to deoxynucleoside monomers. As solid support, 500 to 1000 Å controlled pore glass (CPG) support or organic polymer support, such as primer support PS200 (Amersham), can be used. The solid support usually contains the first nucleoside, such as 5′-O-Dimethoxytrityl-N-6-benzoyladenosine, attached via its 3′-end. After cleavage of the 5′-O-Dimethoxytrityl- group with trichloroacetic acid, chain elongation is achieved using e.g. 5′-O-Dimethoxytrityl-N-protected-2′-O-tert butyldimethylsilyl-nucleoside-3′-O-phosphoramidites. After successive repetitive cycles, the completed oligoribonucleotide is cleaved from the support and deprotected by treatment with concentrated ammonia/ethanol (3:1, v:v) for 24 hours at 30°C. The TBDMS blocking group is finally cleaved off using triethylamine/HF. The crude oligoribonucleotides can be purified by ion exchange high pressure liquid chromatography (HPLC), ion-pair reverse phase HPLC, or polyacrylamide gel electrophoresis (PAGE) and characterized by mass spectrometry.

Synthesis of 5′-conjugates is straightforward by coupling a phosphoramidite of the molecule to be ligated to the 5′-hydroxy group of the terminal nucleotide in solid-phase synthesis. A variety of phosphoramidite derivatives of such ligands, such as cholesterol, acridine, biotin, psoralene, ethyleneglycol, or aminoalkyl residues are commercially available. Alternatively, aminoalkyl functions can be introduced during solid-phase synthesis which allows post-synthesis derivatization by activated conjugate molecules, such as active esters, isothiocynates, or iodo-acetamides.

Synthesis of 3′-end conjugates is usually achieved by using the correspondingly modified solid supports, such as e.g. commercially available cholesterol-derivatized solid supports. Conjugation can however also be done at internucleotide linkages, nucleobases or at the ribose residues, such as at the 2′-postion of ribose.

For cyclic oligoribonucleotides, the elongation of the oligonucleotide chain can be carried out on Nucleotide PS solid support (Glen Research) using standard phosphoramidite chemistry. The cyclization reaction is then carried out on the solid support using a phosphotriester coupling procedure (Alazzouzi et al. (1997) *Nucleosides*

Nucleotides 16:1513-14). On final deprotection with ammonium hydroxide, virtually the only product which comes into solution is the desired cyclic oligonucleotide.

Cyclic oligoribonucleotides of the invention include closed circular forms of RNA and can include single-stranded RNA with or without double-stranded RNA. For example, in one embodiment the cyclic oligoribouncleotide includes double-stranded RNA and takes on a dumbbell conformation with two single-stranded loops connected by an intervening double-stranded segment. Covalently closed, dumbbell-shaped CpG oligodeoxynucleotides have been described in U.S. Pat. No. 6,849,725. In another embodiment the cyclic oligoribonucleotide includes double-stranded RNA and takes on a conformation with three or more single-stranded loops connected by intervening double-stranded segments. In one embodiment an immunostimulatory RNA motif is located in one or more single-stranded segments.

The immunostimulatory ORN of the invention are useful, alone or in combination with other agents, such as adjuvants. An adjuvant as used herein refers to a substance other than an antigen that enhances immune cell activation in response to an antigen, e.g., a humoral and/or cellular immune response. Adjuvants promote the accumulation and/or activation of accessory cells to enhance antigen-specific immune responses. Adjuvants are used to enhance the efficacy of vaccines, i.e., antigen-containing compositions used to induce protective immunity against the antigen.

Adjuvants can work through two general mechanisms and a given adjuvant or adjuvant formulation may act by one or both mechanisms.

The first mechanism is to physically influence the distribution of the antigen to cells or sites where antigen-specific immune responses develop, and this can be a delivery vehicle that changes the biodistribution of the antigen, including targeting to specific areas or cell types, or creates a depot effect such that the antigen is slowly released in the body, thus prolonging the exposure of immune cells to the antigen.

This class of adjuvants includes but is not limited to alum (e.g., aluminum hydroxide, aluminum phosphate); emulsion-based formulations including water-in-oil or oil-in-water-in emulsions made from either mineral or non-mineral oil. These may be oil-in-water emulsions such as Montanide ISA 720 (Seppic, AirLiquide, Paris, France); MF-59 (a squalene-in-water emulsion stabilized with Span 85 and Tween 80; Chiron Corporation, Emeryville, Calif.); and PROVAX (stabilizing detergent and a micelle-

forming agent; IDEC Pharmaceuticals Corporation, San Diego, Calif.). these may also be water-in-oil emulsions such as Montanide ISA 50 (oily composition of mannide oleate and mineral oil, Seppic) or Montanide ISA 206 (oily composition of mannide oleate and mineral oil, Seppic).

The second adjuvant mechanism is as an immune response modifier or immune stimulatory agent. These result in activation of immune cells to better present, recognize or respond to antigens, and thus the antigen specific responses are enhanced for kinetics, magnitude, phenotype or memory. Immune response modifiers typically act through specific receptors such as Toll-like receptors or one of several other non-TLR pathways (e.g., RIG-I), however the pathways for some is yet unknown. This class of adjuvants includes but is not limited to saponins purified from the bark of the *Q. saponaria* tree, such as QS21 (a glycolipid that elutes in the 21st peak with HPLC fractionation; Antigenics, Inc., Worcester, Mass.); poly[di(carboxylatophenoxy)phosphazene (PCPP polymer; Virus Research Institute, USA), Flt3 ligand and Leishmania elongation factor (a purified Leishmania protein; Corixa Corporation, Seattle, Wash.). There are many adjuvants that act through the TLR. Adjuvants that act through TLR4 include derivatives of lipopolysaccharides such as monophosphoryl lipid A (MPL; Ribi ImmunoChem Research, Inc., Hamilton, Mont.) and muramyl dipeptide (MDP; Ribi) andthreonyl-muramyl dipeptide (t-MDP; Ribi); OM-174 (a glucosamine disaccharide related to lipid A; OM Pharma SA, Meyrin, Switzerland). Flagellin is an adjuvant that acts through TLR5. Double stranded RNA acts through TLR3. Adjuvants acting through TLR7 and/or TLR8 include single stranded RNA or oligoribonucleotides (ORN) and synthetic low molecular weight compounds that recognize and activate the TLR including imidazoquinolinamines (e.g., imiquimod, resiquimod; 3M). Adjuvants acting through TLR9 include DNA of viral or bacterial origin, or synthetic oligodeoxynucleotides (ODN), such as CpG ODN.

Adjuvants that have both a physical effect and an immune stimulatory effect are those compounds which have both of the above-identified functions. This class of adjuvants includes but is not limited to ISCOMS (immunostimulating complexes which contain mixed saponins, lipids and form virus-sized particles with pores that can hold antigen; CSL, Melbourne, Australia), Pam3Cys, SB-AS2 (SmithKline Beecham adjuvant system #2 which is an oil-in-water emulsion containing MPL and QS21: SmithKline

Beecham Biologicals [SBB], Rixensart, Belgium), SB-AS4 (SmithKline Beecham adjuvant system #4 which contains alum and MPL; SBB, Belgium), non-ionic block copolymers that form micelles such as CRL 1005 (these contain a linear chain of hydrophobic polyoxypropylene flanked by chains of polyoxyethylene, Vaxcel, Inc., Norcross, Ga.), and Syntex Adjuvant Formulation (SAF, an oil-in-water emulsion containing Tween 80 and a nonionic block copolymer; Syntex Chemicals, Inc., Boulder, Colo.), Montanide IMS (e.g., IMS 1312, water based nanoparticles combined with a soluble immunostimulant, Seppic) as well as many of the delivery vehicles described below.

Also provided is a composition that includes an immunostimulatory polymer of the invention plus another adjuvant, wherein the other adjuvant is a cationic polysaccharide such as chitosan, or a cationic peptide such as protamine, a polyester, a poly(lactic acid), a poly(glycolic acid), or a copolymer of one or more of the above.

Also provided is a composition that includes an immunostimulatory ORN of the invention plus another adjuvant, wherein the other adjuvant is a cytokine. In one embodiment the composition is a conjugate of the immunostimulatory ORN of the invention and the cytokine.

Cytokines are soluble proteins and glycoproteins produced by many types of cells that mediate inflammatory and immune reactions. Cytokines mediate communication between cells of the immune system, acting locally as well as systemically to recruit cells and to regulate their function and proliferation. Categories of cytokines include mediators and regulators of innate immunity, mediators and regulators of adaptive immunity, and stimulators of hematopoiesis. Included among cytokines are interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, and interleukins 19-32 (IL-19 - IL-32), among others), chemokines (e.g., IP-10, RANTES, MIP-1α, MIP-1β, MIP-3α, MCP-1, MCP-2, MCP-3, MCP-4, eotaxin, I-TAC, and BCA-1, among others), as well as other cytokines including type 1 interferons (e.g., IFN-α and IFN-β), type 2 interferon (e.g., IFN-γ), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and various colony stimulating factors (CSFs), including GM-CSF, G-CSF, and M-CSF.

Also provided is a composition that includes an immunostimulatory ORN of the invention plus an immunostimulatory CpG nucleic acid. In one embodiment the

composition is a conjugate of the immunostimulatory ORN of the invention and the CpG nucleic acid, e.g. a RNA:DNA conjugate.

An immunostimulatory CpG nucleic acid as used herein refers to a natural or synthetic DNA sequence that includes a CpG motif and that stimulates activation or proliferation of cells of the immune system. Immunostimulatory CpG nucleic acids have been described in a number of issued patents, published patent applications, and other publications, including U.S. Pat. Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068. In one embodiment the immunostimulatory CpG nucleic acid is a CpG oligodeoxynucleotide (CpG ODN) 6-100 nucleotides long. In one embodiment the immunostimulatory CpG nucleic acid is a CpG oligodeoxynucleotide (CpG ODN) 8-40 nucleotides long.

In some embodiments the ORN include a CG dinucleotide. In other embodiments the ORN is free of a CG dinucleotide.

Immunostimulatory CpG nucleic acids include different classes of CpG nucleic acids. One class is potent for activating B cells but is relatively weak in inducing IFN-α and NK cell activation; this class has been termed the B class. The B class CpG nucleic acids typically are fully stabilized and include an unmethylated CpG dinucleotide within certain preferred base contexts. See, e.g., U.S. Pat. Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; and 6,339,068. Another class is potent for inducing IFN-α and NK cell activation but is relatively weak at stimulating B cells; this class has been termed the A class. The A class CpG nucleic acids typically have a palindromic phosphodiester CpG dinucleotide-containing sequence of at least 6 nucleotides and stabilized poly-G sequences at either or both the 5' and 3' ends. See, for example, published international patent application WO 01/22990. Yet another class of CpG nucleic acids activates B cells and NK cells and induces IFN-α; this class has been termed the C class. The C class CpG nucleic acids, as first characterized, typically are fully stabilized, include a B class-type sequence and a GC-rich palindrome or near-palindrome. This class has been described in published U.S. patent application 2003/0148976, the entire contents of which are incorporated herein by reference.

Immunostimulatory CpG nucleic acids also include so-called soft and semi-soft CpG nucleic acids, as disclosed in published U.S. patent application 2003/0148976, the entire contents of which is incorporated herein by reference. Such soft and semi-soft

immunostimulatory CpG nucleic acids incorporate a combination of nuclease-resistant and nuclease-sensitive internucleotide linkages, wherein the different types of linkages are positioned according to certain rules.

The invention in one aspect provides a vaccine that includes an immunostimulatory ORN of the invention and an antigen. An "antigen" as used herein refers to any molecule capable of being recognized by a T-cell antigen receptor or B-cell antigen receptor. The term broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens generally include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, glycolipids, polysaccharides, carbohydrates, viruses and viral extracts, and multicellular organisms such as parasites, and allergens. With respect to antigens that are proteins, polypeptides, or peptides, such antigens can include nucleic acid molecules encoding such antigens. Antigens more specifically include, but are not limited to, cancer antigens, which include cancer cells and molecules expressed in or on cancer cells; microbial antigens, which include microbes and molecules expressed in or on microbes; allergens, and other disease-associated molecules such as autoreactive T cells. Accordingly, the invention in certain embodiments provides vaccines for cancers, infectious diseases, allergy, addition, diseases caused by abnormally folded proteins, autoimmune disease, and cholesterol management.

A vaccine against infectious disease can be prophylactic or therapeutic. The antigen in the vaccine can be whole live (attenuated), whole killed/inactivated, recombinant live attenuated, subunit purified, subunit recombinant, or a peptide. The vaccine can further comprise additional adjuvants or combinations of adjuvants. The additional adjuvants can be those that have a depot effect (e.g. alum), and immune modifier (e.g. either another TLR agonist or one that works through a non-TLR pathway), or an adjuvant that has both these effects such as an immune stimulating complex (ISCOM®). Adjuvants are described in more detail below.

A vaccine against cancer can also be prophylactic or therapeutic. The cancer antigen can be whole cell (individual DC vaccine), or one or more polypeptides or peptides. These are typically attached to carrier molecule. The vaccine can further

comprise additional adjuvants or combinations of adjuvants such as those described above. Cancer antigens are discussed in more detail below.

For a vaccine for treating allergy the antigen is the allergen or part of the allergen. The allergen may be either contained within or attached to the delivery vehicle. The allergen may be linked to the immune stimulatory ORN. Allergens are discussed in more detail below.

Vaccines for treating addiction may be useful for treating e.g. nicotine addiction, cocaine addiction, methamphetamine, or heroin addiction. The addictive molecule in these cases is the native molecule or a hapten. "Antigens" for inclusion in vaccines against addiction are typically small molecules and may be conjugated to a carrier protein or other carrier particle, or they may be incorporated into a virus-like particle.

Vaccines to treat diseases caused by abnormally folded proteins may be useful for treating diseases such as transmissible spongiform encephalopathy (a variant of Creutzfeld-Jakob disease). The "antigen" in this case would be the scrapie prion, which could be attached to a carrier protein or a live attenuated vector. One example of a vaccine against Alzheimer's disease would be, for example, a vaccine targeted to the beta-amyloid peptide or protein.

Vaccines to treat autimmune diseases are also provided. These vaccines could be useful for treating autoimmune diseases in which the molecule that the autoimmune cells recognize has been identified. For example, a vaccine against autoreactive T-cells that respond to myelin would be used to treat multiple sclerosis.

Vaccines useful for treating cardiovascular diseases and conditions are also provided. The vaccine may target a molecule known to contribute to the etiology of the disease, such as lipoproteins, cholesterol, and molecules involved in cholesterol metabolism. A vaccine for managing cholesterol would comprise, for example, cholesteryl ester transfer protein (CETP) as an antigen. CETP facilitates the exchange of cholesterol from anti-atherogenic apo A-I–containing HDL particles to the atherogenic apo B–containing VLDL and LDL. Such a vaccine could be used to treat high cholesterol or slow the progression of atherosclerosis. The vaccine may be used to treat other cardiovascular diseases and conditions in which a target molecule is known.

The invention in one aspect provides a use of an immunostimulatory ORN of the invention for the preparation of a medicament for vaccinating a subject.

The invention in one aspect provides a method for preparing a vaccine. The method includes the step of placing an immunostimulatory ORN of the invention in intimate association with an antigen and, optionally, a pharmaceutically acceptable carrier.

In some embodiments the immunostimulatory ORN and the antigen or allergen are conjugated. The antigen and the immunostimulatory ORN may be conjugated directly, or they may be conjugated indirectly by means of a linker.

In various embodiments the antigen is a microbial antigen, a cancer antigen, or an allergen. A "microbial antigen" as used herein is an antigen of a microorganism and includes but is not limited to viruses, bacteria, parasites, and fungi. Such antigens include the intact microorganism as well as natural isolates and fragments or derivatives thereof and also synthetic compounds which are identical to or similar to natural microorganism antigens and induce an immune response specific for that microorganism. A compound is similar to a natural microorganism antigen if it induces an immune response (humoral and/or cellular) to a natural microorganism antigen. Such antigens are used routinely in the art and are well known to those of ordinary skill in the art.

Viruses are small infectious agents which generally contain a nucleic acid core and a protein coat, but are not independently living organisms. Viruses can also take the form of infectious nucleic acids lacking a protein. A virus cannot survive in the absence of a living cell within which it can replicate. Viruses enter specific living cells either by endocytosis or direct injection of DNA (phage) and multiply, causing disease. The multiplied virus can then be released and infect additional cells. Some viruses are DNA-containing viruses and others are RNA-containing viruses. In some aspects, the invention also intends to treat diseases in which prions are implicated in disease progression such as for example bovine spongiform encephalopathy (i.e., mad cow disease, BSE) or scrapie infection in animals, or Creutzfeldt-Jakob disease in humans.

Viruses include, but are not limited to, enteroviruses (including, but not limited to, viruses that the family *picornaviridae*, such as polio virus, Coxsackie virus, echo virus), rotaviruses, adenovirus, and hepatitis virus, such as hepatitis A, B, C D and E. Specific examples of viruses that have been found in humans include but are not limited to: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP;

Picornaviridae (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g., equine encephalitis viruses, rubella viruses); *Flaviviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g., influenza viruses); *Bunyaviridae* (e.g., Hantaan viruses, bunya viruses, phleboviruses and Nairo viruses); *Arenaviridae* (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviurses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvoviridae* (parvoviruses); *Papovaviridae* (papillomaviruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV)); *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); *Iridoviridae* (e.g., African swine fever virus); and other viruses acute laryngotracheobronchitis virus, Alphavirus, Kaposi's sarcoma-associated herpesvirus, Newcastle disease virus, Nipah virus, Norwalk virus, Papillomavirus, parainfluenza virus, avian influenza, SARs virus, West Nile virus.

Bacteria are unicellular organisms which multiply asexually by binary fission. They are classified and named based on their morphology, staining reactions, nutrition and metabolic requirements, antigenic structure, chemical composition, and genetic homology. Bacteria can be classified into three groups based on their morphological forms, spherical (coccus), straight-rod (bacillus) and curved or spiral rod (vibrio, campylobacter, spirillum, and spirochaete). Bacteria are also more commonly characterized based on their staining reactions into two classes of organisms, gram-positive and gram-negative. Gram refers to the method of staining which is commonly performed in microbiology labs. Gram-positive organisms retain the stain following the staining procedure and appear a deep violet color. Gram-negative organisms do not retain the stain but take up the counter-stain and thus appear pink.

Infectious bacteria include, but are not limited to, gram negative and gram positive bacteria. Gram positive bacteria include, but are not limited to *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella*

species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pyloris*, *Borrelia burgdorferi*, *Legionella pneumophilia*, *Mycobacteria sps* (e.g., *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A *Streptococcus*), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic species), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp.*, *Enterococcus sp.*, *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia,* and *Actinomyces israelli.*

Parasites are organisms which depend upon other organisms in order to survive and thus must enter, or infect, another organism to continue their life cycle. The infected organism, i.e., the host, provides both nutrition and habitat to the parasite. Although in its broadest sense the term parasite can include all infectious agents (i.e., bacteria, viruses, fungi, protozoa and helminths), generally speaking, the term is used to refer solely to protozoa, helminths, and ectoparasitic arthropods (e.g., ticks, mites, etc.). Protozoa are single-celled organisms which can replicate both intracellularly and extracellularly, particularly in the blood, intestinal tract or the extracellular matrix of tissues. Helminths are multicellular organisms which almost always are extracellular (an exception being *Trichinella* spp.). Helminths normally require exit from a primary host and transmission into a secondary host in order to replicate. In contrast to these aforementioned classes, ectoparasitic arthropods form a parasitic relationship with the external surface of the host body.

Parasites include intracellular parasites and obligate intracellular parasites. Examples of parasites include but are not limited to *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmdodium vivax*, *Plasmodium knowlesi*, *Babesia microti*, *Babesia divergens*, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Trichinella spiralis*, *Leishmania major*, *Leishmania donovani*, *Leishmania braziliensis*, *Leishmania tropica*, *Trypanosoma gambiense*, *Trypanosoma rhodesiense* and *Schistosoma mansoni.*

Fungi are eukaryotic organisms, only a few of which cause infection in vertebrate mammals. Because fungi are eukaryotic organisms, they differ significantly from prokaryotic bacteria in size, structural organization, life cycle and mechanism of multiplication. Fungi are classified generally based on morphological features, modes of reproduction and culture characteristics. Although fungi can cause different types of disease in subjects, such as respiratory allergies following inhalation of fungal antigens, fungal intoxication due to ingestion of toxic substances, such as *Amanita phalloides* toxin and phallotoxin produced by poisonous mushrooms and aflatoxins, produced by aspergillus species, not all fungi cause infectious disease.

Infectious fungi can cause systemic or superficial infections. Primary systemic infection can occur in normal healthy subjects, and opportunistic infections are most frequently found in immunocompromised subjects. The most common fungal agents causing primary systemic infection include *Blastomyces, Coccidioides*, and *Histoplasma.* Common fungi causing opportunistic infection in immunocompromised or immunosuppressed subjects include, but are not limited to, *Candida albicans, Cryptococcus neoformans*, and various *Aspergillus* species. Systemic fungal infections are invasive infections of the internal organs. The organism usually enters the body through the lungs, gastrointestinal tract, or intravenous catheters. These types of infections can be caused by primary pathogenic fungi or opportunistic fungi.

Superficial fungal infections involve growth of fungi on an external surface without invasion of internal tissues. Typical superficial fungal infections include cutaneous fungal infections involving skin, hair, or nails.

Diseases associated with fungal infection include aspergillosis, blastomycosis, candidiasis, chromoblastomycosis, coccidioidomycosis, cryptococcosis, fungal eye infections, fungal hair, nail, and skin infections, histoplasmosis, lobomycosis, mycetoma, otomycosis, paracoccidioidomycosis, disseminated *Penicillium marneffei*, phaeohyphomycosis, rhinosporidioisis, sporotrichosis, and zygomycosis.

Other medically relevant microorganisms have been described extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference. Each of the foregoing lists is illustrative and is not intended to be limiting.

As used herein, the terms "cancer antigen" and "tumor antigen" are used interchangeably to refer to a compound, such as a peptide, protein, or glycoprotein, which is associated with a tumor or cancer cell and which is capable of provoking an immune response when expressed on the surface of an antigen-presenting cell in the context of a major histocompatibility complex (MHC) molecule. Cancer antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation, and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated *ras* oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses.

Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen PA et al. (1994) *Cancer Res* 54:1055-8, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include but are not limited to antigens that are recombinantly expressed, an immunogenic portion of, or a whole tumor or cancer or cell thereof. Such antigens can be isolated or prepared recombinantly or by any other means known in the art.

Examples of tumor antigens include MAGE, MART-1/Melan-A, gp100, dipeptidyl peptidase IV (DPPIV), adenosine deaminase-binding protein (ADAbp), cyclophilin b, colorectal associated antigen (CRC)--C017-1A/GA733, carcinoembryonic antigen (CEA) and its immunogenic epitopes CAP-1 and CAP-2, etv6, aml1, prostate specific antigen (PSA) and its immunogenic epitopes PSA-1, PSA-2, and PSA-3, prostate-specific membrane antigen (PSMA), T-cell receptor/CD3-zeta chain, MAGE-family of tumor antigens (e.g., MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4

(MAGE-B4), MAGE-C1, MAGE-C2, MAGE-C3, MAGE-C4, MAGE-C5), GAGE-family of tumor antigens (e.g., GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, GAGE-9), BAGE, RAGE, LAGE-1, NAG, GnT-V, MUM-1, CDK4, tyrosinase, p53, MUC family, HER2/neu, p21ras, RCAS1, α-fetoprotein, E-cadherin, α-catenin, β-catenin and γ-catenin, p120ctn, $gp100^{Pmel117}$, PRAME, NY-ESO-1, cdc27, adenomatous polyposis coli protein (APC), fodrin, Connexin 37, Ig-idiotype, p15, gp75, GM2 and GD2 gangliosides, viral products such as human papillomavirus proteins, Smad family of tumor antigens, lmp-1, P1A, EBV-encoded nuclear antigen (EBNA)-1, brain glycogen phosphorylase, SSX-1, SSX-2 (HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7, and c-erbB-2. This list is not meant to be limiting.

An "allergen" as used herein is a molecule capable of provoking an immune response characterized by production of IgE. An allergen is also a substance that can induce an allergic or asthmatic response in a susceptible subject. Thus, in the context of this invention, the term allergen means a specific type of antigen which can trigger an allergic response which is mediated by IgE antibody.

The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g., penicillin). Examples of natural animal and plant allergens include proteins specific to the following genuses: Canis (*Canis familiaris*); Dermatophagoides (e.g., *Dermatophagoides farinae*); Felis (*Felis domesticus*); Ambrosia (*Ambrosia artemisiifolia*); Lolium (e.g., *Lolium perenne* and *Lolium multiflorum*); Cryptomeria (*Cryptomeria japonica*); Alternaria (*Alternaria alternata*); Alder; Alnus (*Alnus gultinosa*); Betula (*Betula verrucosa*); Quercus (*Quercus alba*); Olea (*Olea europa*); Artemisia (*Artemisia vulgaris*); Plantago (e.g., *Plantago lanceolata*); Parietaria (e.g., *Parietaria officinalis* and *Parietaria judaica*); Blattella (e.g., *Blattella germanica*); Apis (e.g., *Apis multiflorum*); Cupressus (e.g., *Cupressus sempervirens*, *Cupressus arizonica* and *Cupressus macrocarpa*); Juniperus (e.g., *Juniperus sabinoides*, *Juniperus virginiana*, *Juniperus communis*, and *Juniperus ashei*); Thuya (e.g., *Thuya orientalis*); Chamaecyparis (e.g., *Chamaecyparis obtusa*); Periplaneta (e.g., *Periplaneta americana*); Agropyron (e.g., *Agropyron repens*); Secale (e.g., *Secale cereale*); Triticum (e.g., *Triticum aestivum*); Dactylis (e.g., *Dactylis glomerata*); Festuca (e.g., *Festuca elatior*); Poa (e.g., *Poa pratensis* and *Poa compressa*); Avena (e.g., *Avena*

sativa); Holcus (e.g., *Holcus lanatus*); Anthoxanthum (e.g., *Anthoxanthum odoratum*); Arrhenatherum (e.g., *Arrhenatherum elatius*); Agrostis (e.g., *Agrostis alba*); Phleum (e.g., *Phleum pratense*); Phalaris (e.g., *Phalaris arundinacea*); Paspalum (e.g., *Paspalum notatum*); Sorghum (e.g., *Sorghum halepensis*); and Bromus (e.g., *Bromus inermis*).

The invention in one aspect provides a conjugate of an immunostimulatory ORN of the invention and an antigen. In one embodiment the immunostimulatory ORN of the invention is covalently linked to the antigen. The covalent linkage between the immunostimulatory ORN and the antigen can be any suitable type of covalent linkage, provided the immunostimulatory ORN and the antigen when so joined retain measurable functional activity of each individual component. In one embodiment the covalent linkage is direct. In another embodiment the covalent linkage is indirect, e.g., through a linker moiety. The covalently linked immunostimulatory ORN and antigen may be processed within a cell to release one from the other. In this way delivery to a cell of either component may be enhanced compared to its delivery if administered as a separate preparatation or separate component. In one embodiment the antigen is an antigen *per se*, i.e., it is a preformed antigen.

In one aspect the invention provides a pharmaceutical composition which includes a composition of the invention, in association with a delivery vehicle. In various embodiments the delivery vehicle can be chosen from a cationic lipid, a liposome, a cochleate, a virosome, an immune-stimulating complex (ISCOM®), a microparticle, a microsphere, a nanosphere, a unilamellar vesicle (LUV), a multilamellar vesicle, an emulsome, and a polycationic peptide, a lipoplexe, a polyplexe, a lipopolyplexe, a water-in-oil (W/O) emulsion, an oil-in-water (O/W) emulsion, a water-in-oil-in water (W/O/W) multiple emulsion, a micro-emulsion, a nano-emulsion, a micelle, a dendrimer, a virosome, a virus-like particle, a polymeric nanoparticle (such as a nanosphere or a nanocapsule), a polymeric microparticle (such as a microsphere or a microcapsule), a chitosan, a cyclodextrin, a niosome, or an ISCOM® and, optionally, a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are discussed below. The pharmaceutical composition of the invention optionally can further include an antigen. The composition of the invention, along with the antigen when present, is brought into physical association with the delivery vehicle using any suitable method. The immunostimulatory

composition can be contained within the delivery vehicle, or it can be present on or in association with a solvent-exposed surface of the delivery vehicle. In one embodiment the immunostimulatory ORN is present on or in association with a solvent-exposed surface of the delivery vehicle, and the antigen, if present, is contained within the delivery vehicle. In another embodiment both the immunostimulatory ORN and the antigen are present on or in association with a solvent-exposed surface of the delivery vehicle. In yet another embodiment the antigen is present on or in association with a solvent-exposed surface of the delivery vehicle, and the immunostimulatory ORN is contained within the delivery vehicle. In yet another embodiment both the immunostimulatory ORN and the antigen, if antigen is included, are contained within the delivery vehicle.

The invention also provides methods for use of the immunostimulatory compositions of the invention. In one aspect the invention provides a method of activating an immune cell. The method according to this aspect of the invention includes the step of contacting an immune cell, *in vitro* or *in vivo*, with an effective amount of a composition of the invention, to activate the immune cell. The composition of the invention can optionally include an antigen. An "immune cell" as used herein refers to any bone marrow-derived cell that can participate in an innate or adaptive immune response. Cells of the immune system include, without limitation, dendritic cells (DC), natural killer (NK) cells, monocytes, macrophages, granulocytes, B lymphocytes, plasma cells, T lymphocytes, and precursor cells thereof. In some embodiments the immune cell is a TLR7 expressing cell.

As used herein, the term "effective amount" refers to that amount of a substance that is necessary or sufficient to bring about a desired biological effect. An effective amount can but need not be limited to an amount administered in a single administration.

As used herein, the term "activate an immune cell" refers to inducing an immune cell to enter an activated state that is associated with an immune response. The term "activate an immune cell" refers both to inducing and augmenting an immune response. As used herein, the term "immune response" refers to any aspect of an innate or adaptive immune response that reflects activation of an immune cell to proliferate, to perform an effector immune function, or to produce a gene product involved in an immune response. Gene products involved in an immune response can include secreted products (e.g.,

antibodies, cytokines, and chemokines) as well as intracellular and cell surface molecules characteristic of immune function (e.g., certain cluster of differentiation (CD) antigens, transcription factors, and gene transcripts). The term "immune response" can be applied to a single cell or to a population of cells.

Production of cytokines can be assessed by any of several methods well known in the art, including biological response assays, enzyme-linked immunosorbent assay (ELISA), intracellular fluorescence-activated cell sorting (FACS) analysis, and reverse transcriptase/polymerase chain reaction (RT-PCR). In one embodiment the immune response involves production of IFN-α.

In one embodiment the immune response involves upregulation of cell surface markers of immune cell activation, such as CD25, CD80, CD86, and CD154. Methods for measuring cell surface expression of such markers are well known in the art and include FACS analysis.

For measurement of immune response in a cell or population of cells, in one embodiment the cell or population of cells expresses TLR7. The cell can express the TLR naturally, or it can be manipulated to express the TLR though introduction into the cell of a suitable expression vector for the TLR. In one embodiment the cell or population of cells is obtained as peripheral blood mononuclear cells (PBMC). In one embodiment the cell or population of cells is obtained as a cell line expressing the TLR. In one embodiment the cell or population of cells is obtained as a transient transfectant expressing the TLR. In one embodiment the cell or population of cells is obtained as a stable transfectant expressing the TLR.

Also for use in measuring an immune response in a cell or population of cells, it may be convenient to introduce into the cell or population of cells a reporter construct that is responsive to intracellular signaling by a TLR. In one embodiment such a reporter is a gene placed under the control of an NF-κB promoter. In one embodiment the gene placed under control of the promoter is luciferase. Under suitable conditions of activation, the luciferase reporter construct is expressed and emits a detectable light signal that may be measured quantitatively using a luminometer. Such reporter constructs and other suitable reporter constructs are commercially available.

The invention also contemplates the use of cell-free methods of detecting TLR activation.

The invention in certain aspects relates to compositions and methods for use in therapy. The immunostimulatory compositions of the invention can be used alone or combined with other therapeutic agents. The immunostimulatory composition and other therapeutic agent may be administered simultaneously or sequentially. When the immunostimulatory composition of the invention and the other therapeutic agent are administered simultaneously, they can be administered in the same or separate formulations, but they are administered at the same time. In addition, when the immunostimulatory composition of the invention and the other therapeutic agent are administered simultaneously, they can be administered via the same or separate routes of administration, but they are administered at the same time. The immunostimulatory composition of the invention and another therapeutic agent are administered sequentially when administration of the immunostimulatory composition of the invention is temporally separated from administration of the other therapeutic agent. The separation in time between the administration of these compounds may be a matter of minutes or it may be longer. In one embodiment the immunostimulatory composition of the invention is administered before administration of the other therapeutic agent. In one embodiment the immunostimulatory composition of the invention is administered after administration of the other therapeutic agent. In addition, when the immunostimulatory composition of the invention and the other therapeutic agent are administered sequentially, they can be administered via the same or separate routes of administration. Other therapeutic agents include but are not limited to adjuvants, antigens, vaccines, and medicaments useful for the treatment of infection, cancer, allergy, and asthma.

In one aspect the invention provides a method of vaccinating a subject. The method according to this aspect of the invention includes the step of administering to the subject an antigen and a composition of the invention. In one embodiment the administering the antigen includes administering a nucleic acid encoding the antigen.

A "subject" as used herein refers to a vertebrate animal. In various embodiments the subject is a human, a non-human primate, or other mammal. In certain embodiments the subject is a mouse, rat, guinea pig, rabbit, cat, dog, pig, sheep, goat, cow, or horse.

For use in the method of vaccinating a subject, the composition of the invention in one embodiment includes an antigen. The antigen can be separate from or covalently linked to a ORN of the invention. In one embodiment the composition of the invention

does not itself include the antigen. In this embodiment the antigen can be administered to the subject either separately from the composition of the invention, or together with the composition of the invention. Administration that is separate includes separate in time, separate in location or route of administration, or separate both in time and in location or route of administration. When the composition of the invention and the antigen are administered separate in time, the antigen can be administered before or after the composition of the invention. In one embodiment the antigen is administered 48 hours to 4 weeks after administration of the composition of the invention. The method also contemplates the administration of one or more booster doses of antigen alone, composition alone, or antigen and composition, following an initial administration of antigen and composition.

It is also contemplated by the invention that a subject can be prepared for a future encounter with an unknown antigen by administering to the subject a composition of the invention, wherein the composition does not include an antigen. According to this embodiment the immune system of the subject is prepared to mount a more vigorous response to an antigen that is later encountered by the subject, for example through environmental or occupational exposure. Such method can be used, for example, for travellers, medical workers, and soldiers likely to be exposed to microbial agents.

In one aspect the invention provides a method of treating a subject having an immune system deficiency. The method according to this aspect of the invention includes the step of administering to the subject an effective amount of a composition of the invention to treat the subject. An "immune system deficiency" as used herein refers to an abnormally depressed ability of an immune system to mount an immune response to an antigen. In one embodiment an immune system deficiency is a disease or disorder in which the subject's immune system is not functioning in normal capacity or in which it would be useful to boost the subject's immune response, for example to eliminate a tumor or cancer or an infection in the subject. A "subject having an immune deficiency" as used herein refers to a subject in which there is a depressed ability of the subject's immune system to mount an immune response to an antigen. Subjects having an immune deficiency include subjects having an acquired immune deficiency as well as subjects having a congenital immune system deficiency. Subjects having acquired immune deficiency include, without limitation, subjects having a chronic inflammatory

condition, subjects having chronic renal insufficiency or renal failure, subjects having infection, subjects having cancer, subjects receiving immunosuppressive drugs, subjects receiving other immunosuppressive treatment, and subjects with malnutrition. In one embodiment the subject has a suppressed CD4+ T-cell population. In one embodiment the subject has an infection with human immunodeficiency virus (HIV) or has acquired immunodeficiency syndrome (AIDS). The method according to this aspect of the invention thus provides a method for boosting an immune response or boosting the ability to mount an immune response in a subject in need of a more vigorous immune response.

The compositions and methods of the invention can be used alone or in conjunction with other agents and methods useful for the treatment of infection. In one aspect the invention provides a method of treating a subject having an infection. The method according to this aspect of the invention includes the step of administering to a subject having an infection an effective amount of the composition of the invention to treat the subject.

In one aspect the invention provides a method of treating a subject having an infection. The method according to this aspect of the invention includes the step of administering to a subject having an infection an effective amount of the composition of the invention and an infection medicament to treat the subject.

In one aspect the invention provides a use of an immunostimulatory ORN of the invention for the preparation of a medicament for treating an infection in a subject.

In one aspect the invention provides a composition useful for the treatment of infection. The composition according to this aspect includes an immunostimulatory ORN of the invention and an infection medicament.

As used herein, the term "treat" as used in reference to a subject having a disease or condition shall mean to prevent, ameliorate, or eliminate at least one sign or symptom of the disease or condition in the subject.

A "subject having an infection" is a subject that has a disorder arising from the invasion of the subject, superficially, locally, or systemically, by an infectious microorganism. The infectious microorganism can be a virus, bacterium, fungus, or parasite, as described above.

Infection medicaments include but are not limited to anti-bacterial agents, anti-viral agents, anti-fungal agents and anti-parasitic agents. Phrases such as "anti-infective agent", "antibiotic", "anti-bacterial agent", "anti-viral agent", "anti-fungal agent", "anti-parasitic agent" and "parasiticide" have well-established meanings to those of ordinary skill in the art and are defined in standard medical texts. Briefly, anti-bacterial agents kill or inhibit bacteria, and include antibiotics as well as other synthetic or natural compounds having similar functions. Anti-viral agents can be isolated from natural sources or synthesized and are useful for killing or inhibiting viruses. Anti-fungal agents are used to treat superficial fungal infections as well as opportunistic and primary systemic fungal infections. Anti-parasite agents kill or inhibit parasites. Many antibiotics are low molecular weight molecules which are produced as secondary metabolites by cells, such as microorganisms. In general, antibiotics interfere with one or more functions or structures which are specific for the microorganism and which are not present in host cells.

One of the problems with anti-infective therapies is the side effects occurring in the host that is treated with the anti-infective agent. For instance, many anti-infectious agents can kill or inhibit a broad spectrum of microorganisms and are not specific for a particular type of species. Treatment with these types of anti-infectious agents results in the killing of the normal microbial flora living in the host, as well as the infectious microorganism. The loss of the microbial flora can lead to disease complications and predispose the host to infection by other pathogens, since the microbial flora compete with and function as barriers to infectious pathogens. Other side effects may arise as a result of specific or non-specific effects of these chemical entities on non-microbial cells or tissues of the host.

Another problem with widespread use of anti-infectants is the development of antibiotic-resistant strains of microorganisms. Already, vancomycin-resistant *enterococci*, penicillin-resistant *pneumococci*, multi-resistant *S. aureus*, and multi-resistant *tuberculosis* strains have developed and are becoming major clinical problems. Widespread use of anti-infectants will likely produce many antibiotic-resistant strains of bacteria. As a result, new anti-infective strategies will be required to combat these microorganisms.

Antibacterial antibiotics which are effective for killing or inhibiting a wide range of bacteria are referred to as broad-spectrum antibiotics. Other types of antibacterial antibiotics are predominantly effective against the bacteria of the class gram-positive or gram-negative. These types of antibiotics are referred to as narrow-spectrum antibiotics. Other antibiotics which are effective against a single organism or disease and not against other types of bacteria, are referred to as limited-spectrum antibiotics.

Anti-bacterial agents are sometimes classified based on their primary mode of action. In general, anti-bacterial agents are cell wall synthesis inhibitors, cell membrane inhibitors, protein synthesis inhibitors, nucleic acid synthesis or functional inhibitors, and competitive inhibitors. Cell wall synthesis inhibitors inhibit a step in the process of cell wall synthesis, and in general in the synthesis of bacterial peptidoglycan. Cell wall synthesis inhibitors include β-lactam antibiotics, natural penicillins, semi-synthetic penicillins, ampicillin, clavulanic acid, cephalolsporins, and bacitracin.

The β-lactams are antibiotics containing a four-membered β-lactam ring which inhibits the last step of peptidoglycan synthesis. β-lactam antibiotics can be synthesized or natural. The β-lactam antibiotics produced by *penicillium* are the natural penicillins, such as penicillin G or penicillin V. These are produced by fermentation of *Penicillium chrysogenum*. The natural penicillins have a narrow spectrum of activity and are generally effective against *Streptococcus*, *Gonococcus*, and *Staphylococcus*. Other types of natural penicillins, which are also effective against gram-positive bacteria, include penicillins F, X, K, and O.

Semi-synthetic penicillins are generally modifications of the molecule 6-aminopenicillanic acid produced by a mold. The 6-aminopenicillanic acid can be modified by addition of side chains which produce penicillins having broader spectrums of activity than natural penicillins or various other advantageous properties. Some types of semi-synthetic penicillins have broad spectrums against gram-positive and gram-negative bacteria, but are inactivated by penicillinase. These semi-synthetic penicillins include ampicillin, carbenicillin, oxacillin, azlocillin, mezlocillin, and piperacillin. Other types of semi-synthetic penicillins have narrower activities against gram-positive bacteria, but have developed properties such that they are not inactivated by penicillinase. These include, for instance, methicillin, dicloxacillin, and nafcillin. Some of the broad spectrum semi-synthetic penicillins can be used in combination with β-

lactamase inhibitors, such as clavulanic acids and sulbactam. The β-lactamase inhibitors do not have anti-microbial action but they function to inhibit penicillinase, thus protecting the semi-synthetic penicillin from degradation.

Another type of β-lactam antibiotic is the cephalolsporins. They are sensitive to degradation by bacterial β-lactamases, and thus, are not always effective alone. Cephalolsporins, however, are resistant to penicillinase. They are effective against a variety of gram-positive and gram-negative bacteria. Cephalolsporins include, but are not limited to, cephalothin, cephapirin, cephalexin, cefamandole, cefaclor, cefazolin, cefuroxine, cefoxitin, cefotaxime, cefsulodin, cefetamet, cefixime, ceftriaxone, cefoperazone, ceftazidine, and moxalactam.

Bacitracin is another class of antibiotics which inhibit cell wall synthesis, by inhibiting the release of muropeptide subunits or peptidoglycan from the molecule that delivers the subunit to the outside of the membrane. Although bacitracin is effective against gram-positive bacteria, its use is limited in general to topical administration because of its high toxicity.

Carbapenems are another broad-spectrum β-lactam antibiotic, which is capable of inhibiting cell wall synthesis. Examples of carbapenems include, but are not limited to, imipenems. Monobactams are also broad-spectrum β-lactam antibiotics, and include, euztreonam. An antibiotic produced by *Streptomyces*, vancomycin, is also effective against gram-positive bacteria by inhibiting cell membrane synthesis.

Another class of anti-bacterial agents is the anti-bacterial agents that are cell membrane inhibitors. These compounds disorganize the structure or inhibit the function of bacterial membranes. One problem with anti-bacterial agents that are cell membrane inhibitors is that they can produce effects in eukaryotic cells as well as bacteria because of the similarities in phospholipids in bacterial and eukaryotic membranes. Thus these compounds are rarely specific enough to permit these compounds to be used systemically and prevent the use of high doses for local administration.

One clinically useful cell membrane inhibitor is Polymyxin. Polymyxins interfere with membrane function by binding to membrane phospholipids. Polymyxin is effective mainly against Gram-negative bacteria and is generally used in severe *Pseudomonas* infections or *Pseudomonas* infections that are resistant to less toxic

antibiotics. The severe side effects associated with systemic administration of this compound include damage to the kidney and other organs.

Other cell membrane inhibitors include Amphotericin B and Nystatin which are anti-fungal agents used predominantly in the treatment of systemic fungal infections and *Candida* yeast infections. Imidazoles are another class of antibiotic that is a cell membrane inhibitor. Imidazoles are used as anti-bacterial agents as well as anti-fungal agents, e.g., used for treatment of yeast infections, dermatophytic infections, and systemic fungal infections. Imidazoles include but are not limited to clotrimazole, miconazole, ketoconazole, itraconazole, and fluconazole.

Many anti-bacterial agents are protein synthesis inhibitors. These compounds prevent bacteria from synthesizing structural proteins and enzymes and thus cause inhibition of bacterial cell growth or function or cell death. In general these compounds interfere with the processes of transcription or translation. Anti-bacterial agents that block transcription include but are not limited to Rifampins and Ethambutol. Rifampins, which inhibit the enzyme RNA polymerase, have a broad spectrum activity and are effective against gram-positive and gram-negative bacteria as well as *Mycobacterium tuberculosis*. Ethambutol is effective against *Mycobacterium tuberculosis*.

Anti-bacterial agents which block translation interfere with bacterial ribosomes to prevent mRNA from being translated into proteins. In general this class of compounds includes but is not limited to tetracyclines, chloramphenicol, the macrolides (e.g., erythromycin) and the aminoglycosides (e.g., streptomycin).

The aminoglycosides are a class of antibiotics which are produced by the bacterium *Streptomyces,* such as, for instance streptomycin, kanamycin, tobramycin, amikacin, and gentamicin. Aminoglycosides have been used against a wide variety of bacterial infections caused by Gram-positive and Gram-negative bacteria. Streptomycin has been used extensively as a primary drug in the treatment of *tuberculosis*. Gentamicin is used against many strains of Gram-positive and Gram-negative bacteria, including *Pseudomonas* infections, especially in combination with Tobramycin. Kanamycin is used against many Gram-positive bacteria, including penicillin-resistant *Staphylococci*. One side effect of aminoglycosides that has limited their use clinically is that at dosages which are essential for efficacy, prolonged use has been shown to impair kidney function and cause damage to the auditory nerves leading to deafness.

Another type of translation inhibitor anti-bacterial agent is the tetracyclines. The tetracyclines are a class of antibiotics that are broad-spectrum and are effective against a variety of gram-positive and gram-negative bacteria. Examples of tetracyclines include tetracycline, minocycline, doxycycline, and chlortetracycline. They are important for the treatment of many types of bacteria but are particularly important in the treatment of Lyme disease. As a result of their low toxicity and minimal direct side effects, the tetracyclines have been overused and misused by the medical community, leading to problems. For instance, their overuse has led to widespread development of resistance.

Anti-bacterial agents such as the macrolides bind reversibly to the 50 S ribosomal subunit and inhibit elongation of the protein by peptidyl transferase or prevent the release of uncharged tRNA from the bacterial ribosome or both. These compounds include erythromycin, roxithromycin, clarithromycin, oleandomycin, and azithromycin. Erythromycin is active against most Gram-positive bacteria, *Neisseria*, *Legionella* and *Haemophilus*, but not against the *Enterobacteriaceae*. Lincomycin and clindamycin, which block peptide bond formation during protein synthesis, are used against gram-positive bacteria.

Another type of translation inhibitor is chloramphenicol. Chloramphenicol binds the 70 S ribosome inhibiting the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis. One serious side effect associated with chloramphenicol is aplastic anemia. Aplastic anemia develops at doses of chloramphenicol which are effective for treating bacteria in a small proportion (1/50,000) of patients. Chloramphenicol which was once a highly prescribed antibiotic is now seldom uses as a result of the deaths from anemia. Because of its effectiveness it is still used in life-threatening situations (e.g., typhoid fever).

Some anti-bacterial agents disrupt nucleic acid synthesis or function, e.g., bind to DNA or RNA so that their messages cannot be read. These include but are not limited to quinolones and co-trimoxazole, both synthetic chemicals and rifamycins, a natural or semi-synthetic chemical. The quinolones block bacterial DNA replication by inhibiting the DNA gyrase, the enzyme needed by bacteria to produce their circular DNA. They are broad spectrum and examples include norfloxacin, ciprofloxacin, enoxacin, nalidixic acid and temafloxacin. Nalidixic acid is a bactericidal agent that binds to the DNA gyrase enzyme (topoisomerase) which is essential for DNA replication and allows

supercoils to be relaxed and reformed, inhibiting DNA gyrase activity. The main use of nalidixic acid is in treatment of lower urinary tract infections (UTI) because it is effective against several types of Gram-negative bacteria such as *E. coli*, *Enterobacter aerogenes*, *K. pneumoniae* and *Proteus* species which are common causes of UTI. Co-trimoxazole is a combination of sulfamethoxazole and trimethoprim, which blocks the bacterial synthesis of folic acid needed to make DNA nucleotides. Rifampicin is a derivative of rifamycin that is active against Gram-positive bacteria (including *Mycobacterium tuberculosis* and meningitis caused by *Neisseria meningitidis*) and some Gram-negative bacteria. Rifampicin binds to the beta subunit of the polymerase and blocks the addition of the first nucleotide which is necessary to activate the polymerase, thereby blocking mRNA synthesis.

Another class of anti-bacterial agents is compounds that function as competitive inhibitors of bacterial enzymes. The competitive inhibitors are mostly all structurally similar to a bacterial growth factor and compete for binding but do not perform the metabolic function in the cell. These compounds include sulfonamides and chemically modified forms of sulfanilamide which have even higher and broader antibacterial activity. The sulfonamides (e.g., gantrisin and trimethoprim) are useful for the treatment of *Streptococcus pneumoniae*, beta-hemolytic *streptococci* and *E. coli*, and have been used in the treatment of uncomplicated UTI caused by *E. coli*, and in the treatment of meningococcal meningitis.

Anti-viral agents are compounds which prevent infection of cells by viruses or replication of the virus within the cell. There are many fewer antiviral drugs than antibacterial drugs because the process of viral replication is so closely related to DNA replication within the host cell, that non-specific antiviral agents would often be toxic to the host. There are several stages within the process of viral infection which can be blocked or inhibited by antiviral agents. These stages include, attachment of the virus to the host cell (immunoglobulin or binding peptides), uncoating of the virus (e.g. amantadine), synthesis or translation of viral mRNA (e.g. interferon), replication of viral RNA or DNA (e.g. nucleoside analogues), maturation of new virus proteins (e.g. protease inhibitors), and budding and release of the virus.

Another category of anti-viral agents are nucleoside analogues. Nucleoside analogues are synthetic compounds which are similar to nucleosides, but which have an

incomplete or abnormal deoxyribose or ribose group. Once the nucleoside analogues are in the cell, they are phosphorylated, producing the triphosphate form which competes with normal nucleotides for incorporation into the viral DNA or RNA. Once the triphosphate form of the nucleoside analogue is incorporated into the growing nucleic acid chain, it causes irreversible association with the viral polymerase and thus chain termination. Nucleoside analogues include, but are not limited to, acyclovir (used for the treatment of herpes simplex virus and varicella-zoster virus), gancyclovir (useful for the treatment of cytomegalovirus), idoxuridine, ribavirin (useful for the treatment of respiratory syncitial virus), dideoxyinosine, dideoxycytidine, and zidovudine (azidothymidine).

Another class of anti-viral agents includes cytokines such as interferons. The interferons are cytokines which are secreted by virus-infected cells as well as immune cells. The interferons function by binding to specific receptors on cells adjacent to the infected cells, causing the change in the cell which protects it from infection by the virus. α and β-interferon also induce the expression of Class I and Class II MHC molecules on the surface of infected cells, resulting in increased antigen presentation for host immune cell recognition. α and β-interferons are available as recombinant forms and have been used for the treatment of chronic hepatitis B and C infection. At the dosages which are effective for anti-viral therapy, interferons have severe side effects such as fever, malaise and weight loss.

Immunoglobulin therapy is used for the prevention of viral infection. Immunoglobulin therapy for viral infections is different from bacterial infections, because rather than being antigen-specific, the immunoglobulin therapy functions by binding to extracellular virions and preventing them from attaching to and entering cells which are susceptible to the viral infection. The therapy is useful for the prevention of viral infection for the period of time that the antibodies are present in the host. In general there are two types of immunoglobulin therapies, normal immune globulin therapy and hyper-immune globulin therapy. Normal immune globulin therapy utilizes a antibody product which is prepared from the serum of normal blood donors and pooled. This pooled product contains low titers of antibody to a wide range of human viruses, such as hepatitis A, parvovirus, enterovirus (especially in neonates). Hyper-immune globulin therapy utilizes antibodies which are prepared from the serum of individuals who have

high titers of an antibody to a particular virus. Those antibodies are then used against a specific virus. Examples of hyper-immune globulins include zoster immune globulin (useful for the prevention of varicella in immunocompromised children and neonates), human rabies immune globulin (useful in the post-exposure prophylaxis of a subject bitten by a rabid animal), hepatitis B immune globulin (useful in the prevention of hepatitis B virus, especially in a subject exposed to the virus), and RSV immune globulin (useful in the treatment of respiratory syncitial virus infections).

Anti-fungal agents are useful for the treatment and prevention of infective fungi. Anti-fungal agents are sometimes classified by their mechanism of action. Some anti-fungal agents function as cell wall inhibitors by inhibiting glucose synthase. These include, but are not limited to, basiungin/ECB. Other anti-fungal agents function by destabilizing membrane integrity. These include, but are not limited to, imidazoles, such as clotrimazole, sertaconzole, fluconazole, itraconazole, ketoconazole, miconazole, and voriconacole, as well as FK 463, amphotericin B, BAY 38-9502, MK 991, pradimicin, UK 292, butenafine, and terbinafine. Other anti-fungal agents function by breaking down chitin (e.g., chitinase) or immunosuppression (501 cream).

Parasiticides are agents that kill parasites directly. Such compounds are known in the art and are generally commercially available. Examples of parasiticides useful for human administration include but are not limited to albendazole, amphotericin B, benznidazole, bithionol, chloroquine HCl, chloroquine phosphate, clindamycin, dehydroemetine, diethylcarbamazine, diloxanide furoate, eflornithine, furazolidaone, glucocorticoids, halofantrine, iodoquinol, ivermectin, mebendazole, mefloquine, meglumine antimoniate, melarsoprol, metrifonate, metronidazole, niclosamide, nifurtimox, oxamniquine, paromomycin, pentamidine isethionate, piperazine, praziquantel, primaquine phosphate, proguanil, pyrantel pamoate, pyrimethanmine-sulfonamides, pyrimethanmine-sulfadoxine, quinacrine HCl, quinine sulfate, quinidine gluconate, spiramycin, stibogluconate sodium (sodium antimony gluconate), suramin, tetracycline, doxycycline, thiabendazole, tinidazole, trimethroprim-sulfamethoxazole, and tryparsamide.

The ORNs are also useful for treating and preventing autoimmune disease. Autoimmune disease is a class of diseases in which an subject's own antibodies react with host tissue or in which immune effector T cells are autoreactive to endogenous self

peptides and cause destruction of tissue. Thus an immune response is mounted against a subject's own antigens, referred to as self antigens. Autoimmune diseases include but are not limited to rheumatoid arthritis, Crohn's disease, multiple sclerosis, systemic lupus erythematosus (SLE), autoimmune encephalomyelitis, myasthenia gravis (MG), Hashimoto's thyroiditis, Goodpasture's syndrome, pemphigus (e.g., pemphigus vulgaris), Grave's disease, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, scleroderma with anti-collagen antibodies, mixed connective tissue disease, polymyositis, pernicious anemia, idiopathic Addison's disease, autoimmune-associated infertility, glomerulonephritis (e.g., crescentic glomerulonephritis, proliferative glomerulonephritis), bullous pemphigoid, Sjögren's syndrome, insulin resistance, and autoimmune diabetes mellitus.

A "self-antigen" as used herein refers to an antigen of a normal host tissue. Normal host tissue does not include cancer cells. Thus an immune response mounted against a self-antigen, in the context of an autoimmune disease, is an undesirable immune response and contributes to destruction and damage of normal tissue, whereas an immune response mounted against a cancer antigen is a desirable immune response and contributes to the destruction of the tumor or cancer. Thus, in some aspects of the invention aimed at treating autoimmune disorders it is not recommended that the ORN be administered with self antigens, particularly those that are the targets of the autoimmune disorder.

In other instances, the ORN may be delivered with low doses of self-antigens. A number of animal studies have demonstrated that mucosal administration of low doses of antigen can result in a state of immune hyporesponsiveness or "tolerance." The active mechanism appears to be a cytokine-mediated immune deviation away from a Th1 towards a predominantly Th2 and Th3 (i.e., TGF-β dominated) response. The active suppression with low dose antigen delivery can also suppress an unrelated immune response (bystander suppression) which is of considerable interest in the therapy of autoimmune diseases, for example, rheumatoid arthritis and SLE. Bystander suppression involves the secretion of Th1-counter-regulatory, suppressor cytokines in the local environment where proinflammatory and Th1 cytokines are released in either an antigen-specific or antigen-nonspecific manner. "Tolerance" as used herein is used to refer to this phenomenon. Indeed, oral tolerance has been effective in the treatment of a number

of autoimmune diseases in animals including: experimental autoimmune encephalomyelitis (EAE), experimental autoimmune myasthenia gravis, collagen-induced arthritis (CIA), and insulin-dependent diabetes mellitus. In these models, the prevention and suppression of autoimmune disease is associated with a shift in antigen-specific humoral and cellular responses from a Th1 to Th2/Th3 response.

The compositions and methods of the invention can be used alone or in conjunction with other agents and methods useful for the treatment of cancer. In one aspect the invention provides a method of treating a subject having a cancer. The method according to this aspect of the invention includes the step of administering to a subject having a cancer an effective amount of a composition of the invention to treat the subject.

In one aspect the invention provides a method of treating a subject having a cancer. The method according to this aspect of the invention includes the step of administering to a subject having a cancer an effective amount of the composition of the invention and an anti-cancer therapy to treat the subject.

In one aspect the invention provides a use of an immunostimulatory ORN of the invention for the preparation of a medicament for treating cancer in a subject.

In one aspect the invention provides a composition useful for the treatment of cancer. The composition according to this aspect includes an immunostimulatory ORN of the invention and a cancer medicament.

A subject having a cancer is a subject that has detectable cancerous cells. The cancer may be a malignant or non-malignant cancer. "Cancer" as used herein refers to an uncontrolled growth of cells which interferes with the normal functioning of the bodily organs and systems. Cancers which migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. Hemopoietic cancers, such as leukemia, are able to outcompete the normal hemopoietic compartments in a subject, thereby leading to hemopoietic failure (in the form of anemia, thrombocytopenia and neutropenia) ultimately causing death.

A metastasis is a region of cancer cells, distinct from the primary tumor location, resulting from the dissemination of cancer cells from the primary tumor to other parts of the body. At the time of diagnosis of the primary tumor mass, the subject may be

monitored for the presence of metastases. Metastases are most often detected through the sole or combined use of magnetic resonance imaging (MRI) scans, computed tomography (CT) scans, blood and platelet counts, liver function studies, chest X-rays and bone scans in addition to the monitoring of specific symptoms.

Cancers include, but are not limited to, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and central nervous system (CNS) cancer; breast cancer; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; intra-epithelial neoplasm; kidney cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g. small cell and non-small cell); lymphoma including Hodgkin's and Non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (e.g., lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; sarcoma; skin cancer; stomach cancer; testicular cancer; thyroid cancer; uterine cancer; cancer of the urinary system, as well as other carcinomas, adenocarcinomas, and sarcomas.

The immunostimulatory composition of the invention may also be administered in conjunction with an anti-cancer therapy. Anti-cancer therapies include cancer medicaments, radiation, and surgical procedures. As used herein, a "cancer medicament" refers to an agent which is administered to a subject for the purpose of treating a cancer. As used herein, "treating cancer" includes preventing the development of a cancer, reducing the symptoms of cancer, and/or inhibiting the growth of an established cancer. In other aspects, the cancer medicament is administered to a subject at risk of developing a cancer for the purpose of reducing the risk of developing the cancer. Various types of medicaments for the treatment of cancer are described herein. For the purpose of this specification, cancer medicaments are classified as chemotherapeutic agents, immunotherapeutic agents, cancer vaccines, hormone therapy, and biological response modifiers.

The chemotherapeutic agent may be selected from the group consisting of methotrexate, vincristine, adriamycin, cisplatin, non-sugar containing chloroethylnitrosoureas, 5-fluorouracil, mitomycin C, bleomycin, doxorubicin, dacarbazine, taxol, fragyline, Meglamine GLA, valrubicin, carmustaine and poliferposan,

MMI270, BAY 12-9566, RAS famesyl transferase inhibitor, famesyl transferase inhibitor, MMP, MTA/LY231514, LY264618/Lometexol, Glamolec, CI-994, TNP-470, Hycamtin/Topotecan, PKC412, Valspodar/PSC833, Novantrone/Mitroxantrone, Metaret/Suramin, Batimastat, E7070, BCH-4556, CS-682, 9-AC, AG3340, AG3433, Incel/VX-710, VX-853, ZD0101, ISI641, ODN 698, TA 2516/Marmistat, BB2516/Marmistat, CDP 845, D2163, PD183805, DX8951f, Lemonal DP 2202, FK 317, Picibanil/OK-432, AD 32/Valrubicin, Metastron/strontium derivative, Temodal/Temozolomide, Evacet/liposomal doxorubicin, Yewtaxan/Paclitaxel, Taxol/Paclitaxel, Xeload/Capecitabine, Furtulon/Doxifluridine, Cyclopax/oral paclitaxel, Oral Taxoid, SPU-077/Cisplatin, HMR 1275/Flavopiridol, CP-358 (774)/EGFR, CP-609 (754)/RAS oncogene inhibitor, BMS-182751/oral platinum, UFT(Tegafur/Uracil), Ergamisol/Levamisole, Eniluracil/776C85/5FU enhancer, Campto/Levamisole, Camptosar/Irinotecan, Tumodex/Ralitrexed, Leustatin/Cladribine, Paxex/Paclitaxel, Doxil/liposomal doxorubicin, Caelyx/liposomal doxorubicin, Fludara/Fludarabine, Pharmarubicin/Epirubicin, DepoCyt, ZD1839, LU 79553/Bis-Naphtalimide, LU 103793/Dolastain, Caetyx/liposomal doxorubicin, Gemzar/Gemcitabine, ZD 0473/Anormed, YM 116, Iodine seeds, CDK4 and CDK2 inhibitors, PARP inhibitors, D4809/Dexifosamide, Ifes/Mesnex/Ifosamide, Vumon/Teniposide, Paraplatin/Carboplatin, Plantinol/cisplatin, Vepeside/Etoposide, ZD 9331, Taxotere/Docetaxel, prodrug of guanine arabinoside, Taxane Analog, nitrosoureas, alkylating agents such as melphelan and cyclophosphamide, Aminoglutethimide, Asparaginase, Busulfan, Carboplatin, Chlorombucil, Cytarabine HCI, Dactinomycin, Daunorubicin HCl, Estramustine phosphate sodium, Etoposide (VP16-213), Floxuridine, Fluorouracil (5-FU), Flutamide, Hydroxyurea (hydroxycarbamide), Ifosfamide, Interferon Alfa-2a, Alfa-2b, Leuprolide acetate (LHRH-releasing factor analogue), Lomustine (CCNU), Mechlorethamine HCl (nitrogen mustard), Mercaptopurine, Mesna, Mitotane (o.p′-DDD), Mitoxantrone HCl, Octreotide, Plicamycin, Procarbazine HCl, Streptozocin, Tamoxifen citrate, Thioguanine, Thiotepa, Vinblastine sulfate, Amsacrine (m-AMSA), Azacitidine, Erthropoietin, Hexamethylmelamine (HMM), Interleukin 2, Mitoguazone (methyl-GAG; methyl glyoxal bis-guanylhydrazone; MGBG), Pentostatin (2'deoxycoformycin), Semustine (methyl-CCNU), Teniposide (VM-26) and Vindesine sulfate, but it is not so limited.

The immunotherapeutic agent may be selected from the group consisting of 3622W94, 4B5, ANA Ab, anti-FLK-2, anti-VEGF, ATRAGEN, AVASTIN (bevacizumab; Genentech), BABS, BEC2, BEXXAR (tositumomab; GlaxoSmithKline), C225, CAMPATH (alemtuzumab; Genzyme Corp.), CEACIDE, CMA 676, EMD-72000, ERBITUX (cetuximab; ImClone Systems, Inc.), Gliomab-H, GNI-250, HERCEPTIN (trastuzumab; Genentech), IDEC-Y2B8, ImmuRAIT-CEA, ior c5, ior egf.r3, ior t6, LDP-03, LymphoCide, MDX-11, MDX-22, MDX-210, MDX-220, MDX-260, MDX-447, MELIMMUNE-1, MELIMMUNE-2, Monopharm-C, NovoMAb-G2, Oncolym, OV103, Ovarex, Panorex, Pretarget, Quadramet, Ributaxin, RITUXAN (rituximab; Genentech), SMART 1D10 Ab, SMART ABL 364 Ab,, SMART M195, TNT, and ZENAPAX (daclizumab; Roche), but it is not so limited.

The cancer vaccine may be selected from the group consisting of EGF, Anti-idiotypic cancer vaccines, Gp75 antigen, GMK melanoma vaccine, MGV ganglioside conjugate vaccine, Her2/neu, Ovarex, M-Vax, O-Vax, L-Vax, STn-KHL theratope, BLP25 (MUC-1), liposomal idiotypic vaccine, Melacine, peptide antigen vaccines, toxin/antigen vaccines, MVA-based vaccine, PACIS, BCG vacine, TA-HPV, TA-CIN, DISC-virus and ImmuCyst/TheraCys, but it is not so limited.

The compositions and methods of the invention can be used alone or in conjunction with other agents and methods useful for the treatment of allergy. In one aspect the invention provides a method of treating a subject having an allergic condition. The method according to this aspect of the invention includes the step of administering to a subject having an allergic condition an effective amount of a composition of the invention to treat the subject.

In one aspect the invention provides a method of treating a subject having an allergic condition. The method according to this aspect of the invention includes the step of administering to a subject having an allergic condition an effective amount of the composition of the invention and an anti-allergy therapy to treat the subject.

In one aspect the invention provides a use of an immunostimulatory ORN of the invention for the preparation of a medicament for treating an allergic condition in a subject.

In one aspect the invention provides a composition useful for the treatment of an allergic condition. The composition according to this aspect includes an immunostimulatory ORN of the invention and an allergy medicament.

A "subject having an allergic condition" shall refer to a subject that is currently experiencing or has previously experienced an allergic reaction in response to an allergen. An "allergic condition" or "allergy" refers to acquired hypersensitivity to a substance (allergen). Allergic conditions include but are not limited to eczema, allergic rhinitis or coryza, hay fever, allergic conjunctivitis, bronchial asthma, urticaria (hives) and food allergies, other atopic conditions including atopic dermatitis; anaphylaxis; drug allergy; and angioedema.

Allergy is typically an episodic condition associated with the production of antibodies from a particular class of immunoglobulin, IgE, against allergens. The development of an IgE-mediated response to common aeroallergens is also a factor which indicates predisposition towards the development of asthma. If an allergen encounters a specific IgE which is bound to an IgE Fc receptor (FcεR) on the surface of a basophil (circulating in the blood) or mast cell (dispersed throughout solid tissue), the cell becomes activated, resulting in the production and release of mediators such as histamine, serotonin, and lipid mediators.

An allergic reaction occurs when tissue-sensitizing immunoglobulin of the IgE type reacts with foreign allergen. The IgE antibody is bound to mast cells and/or basophils, and these specialized cells release chemical mediators (vasoactive amines) of the allergic reaction when stimulated to do so by allergens bridging the ends of the antibody molecule. Histamine, platelet activating factor, arachidonic acid metabolites, and serotonin are among the best known mediators of allergic reactions in man. Histamine and the other vasoactive amines are normally stored in mast cells and basophil leukocytes. The mast cells are dispersed throughout animal tissue and the basophils circulate within the vascular system. These cells manufacture and store histamine within the cell unless the specialized sequence of events involving IgE binding occurs to trigger its release.

Symptoms of an allergic reaction vary, depending on the location within the body where the IgE reacts with the antigen. If the reaction occurs along the respiratory epithelium, the symptoms generally are sneezing, coughing and asthmatic reactions. If

the interaction occurs in the digestive tract, as in the case of food allergies, abdominal pain and diarrhea are common. Systemic allergic reactions, for example following a bee sting or administration of penicillin to an allergic subject, can be severe and often life-threatening.

Allergy is associated with a Th2-type of immune response, which is characterized at least in part by Th2 cytokines IL-4 and IL-5, as well as antibody isotype switching to IgE. Th1 and Th2 immune responses are mutually counter-regulatory, so that skewing of the immune response toward a Th1-type of immune response can prevent or ameliorate a Th2-type of immune response, including allergy. The immunostimulatory ORN of the invention are therefore useful by themselves to treat a subject having an allergic condition because the immunostimulatory ORN can skew the immune response toward a Th1-type of immune response. Alternatively or in addition, the immunostimulatory ORN of the invention can be used in combination with an allergen to treat a subject having an allergic condition.

The immunostimulatory composition of the invention may also be administered in conjunction with an anti-allergy therapy. Conventional methods for treating or preventing allergy have involved the use of allergy medicaments or desensitization therapies. Some evolving therapies for treating or preventing allergy include the use of neutralizing anti-IgE antibodies. Anti-histamines and other drugs which block the effects of chemical mediators of the allergic reaction help to regulate the severity of the allergic symptoms but do not prevent the allergic reaction and have no effect on subsequent allergic responses. Desensitization therapies are performed by giving small doses of an allergen, usually by injection under the skin, in order to induce an IgG-type response against the allergen. The presence of IgG antibody helps to neutralize the production of mediators resulting from the induction of IgE antibodies, it is believed. Initially, the subject is treated with a very low dose of the allergen to avoid inducing a severe reaction and the dose is slowly increased. This type of therapy is dangerous because the subject is actually administered the compounds which cause the allergic response and severe allergic reactions can result.

Allergy medicaments include, but are not limited to, anti-histamines, corticosteroids, and prostaglandin inducers. Anti-histamines are compounds which counteract histamine released by mast cells or basophils. These compounds are well

known in the art and commonly used for the treatment of allergy. Anti-histamines include, but are not limited to, acrivastine, astemizole, azatadine, azelastine, betatastine, brompheniramine, buclizine, cetirizine, cetirizine analogues, chlorpheniramine, clemastine, CS 560, cyproheptadine, desloratadine, dexchlorpheniramine, ebastine, epinastine, fexofenadine, HSR 609, hydroxyzine, levocabastine, loratidine, methscopolamine, mizolastine, norastemizole, phenindamine, promethazine, pyrilamine, terfenadine, and tranilast.

Corticosteroids include, but are not limited to, methylprednisolone, prednisolone, prednisone, beclomethasone, budesonide, dexamethasone, flunisolide, fluticasone propionate, and triamcinolone. Although dexamethasone is a corticosteroid having anti-inflammatory action, it is not regularly used for the treatment of allergy or asthma in an inhaled form because it is highly absorbed and it has long-term suppressive side effects at an effective dose. Dexamethasone, however, can be used according to the invention for treating allergy or asthma because when administered in combination with a composition of the invention it can be administered at a low dose to reduce the side effects. Some of the side effects associated with corticosteroid use include cough, dysphonia, oral thrush (candidiasis), and in higher doses, systemic effects, such as adrenal suppression, glucose intolerance, osteoporosis, aseptic necrosis of bone, cataract formation, growth suppression, hypertension, muscle weakness, skin thinning, and easy bruising. Barnes & Peterson (1993) *Am Rev Respir Dis* 148:S1-S26; and Kamada AK et al. (1996) *Am J Respir Crit Care Med* 153:1739-48.

The compositions and methods of the invention can be used alone or in conjunction with other agents and methods useful for the treatment of asthma. In one aspect the invention provides a method of treating a subject having asthma. The method according to this aspect of the invention includes the step of administering to a subject having asthma an effective amount of a composition of the invention to treat the subject.

In one aspect the invention provides a method of treating a subject having asthma. The method according to this aspect of the invention includes the step of administering to a subject having asthma an effective amount of the composition of the invention and an anti-asthma therapy to treat the subject.

In one aspect the invention provides a use of an immunostimulatory ORN of the invention for the preparation of a medicament for treating asthma in a subject.

In one aspect the invention provides a composition useful for the treatment of asthma. The composition according to this aspect includes an immunostimulatory ORN of the invention and an asthma medicament.

"Asthma" as used herein refers to a disorder of the respiratory system characterized by inflammation and narrowing of the airways, and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively, associated with an atopic or allergic condition. Symptoms of asthma include recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, resulting from airflow obstruction. Airway inflammation associated with asthma can be detected through observation of a number of physiological changes, such as, denudation of airway epithelium, collagen deposition beneath basement membrane, edema, mast cell activation, inflammatory cell infiltration, including neutrophils, eosinophils, and lymphocytes. As a result of the airway inflammation, asthma patients often experience airway hyper-responsiveness, airflow limitation, respiratory symptoms, and disease chronicity. Airflow limitations include acute bronchoconstriction, airway edema, mucous plug formation, and airway remodeling, features which often lead to bronchial obstruction. In some cases of asthma, sub-basement membrane fibrosis may occur, leading to persistent abnormalities in lung function.

Research over the past several years has revealed that asthma likely results from complex interactions among inflammatory cells, mediators, and other cells and tissues resident in the airways. Mast cells, eosinophils, epithelial cells, macrophage, and activated T cells all play an important role in the inflammatory process associated with asthma. Djukanovic R et al. (1990) *Am Rev Respir Dis* 142:434-457. It is believed that these cells can influence airway function through secretion of preformed and newly synthesized mediators which can act directly or indirectly on the local tissue. It has also been recognized that subpopulations of T lymphocytes (Th2) play an important role in regulating allergic inflammation in the airway by releasing selective cytokines and establishing disease chronicity. Robinson DS et al. (1992) *N Engl J Med* 326:298-304.

Asthma is a complex disorder which arises at different stages in development and can be classified based on the degree of symptoms as acute, subacute, or chronic. An acute inflammatory response is associated with an early recruitment of cells into the airway. The subacute inflammatory response involves the recruitment of cells as well as

the activation of resident cells causing a more persistent pattern of inflammation. Chronic inflammatory response is characterized by a persistent level of cell damage and an ongoing repair process, which may result in permanent abnormalities in the airway.

A "subject having asthma" is a subject that has a disorder of the respiratory system characterized by inflammation and narrowing of the airways and increased reactivity of the airways to inhaled agents. Factors associated with initiation of asthma include, but are not limited to, allergens, cold temperature, exercise, viral infections, and SO_2.

As mentioned above, asthma may be associated with a Th2-type of immune response, which is characterized at least in part by Th2 cytokines IL-4 and IL-5, as well as antibody isotype switching to IgE. Th1 and Th2 immune responses are mutually counter-regulatory, so that skewing of the immune response toward a Th1-type of immune response can prevent or ameliorate a Th2-type of immune response, including allergy. The modified oligoribonucleotide analogs of the invention are therefore useful by themselves to treat a subject having asthma because the analogs can skew the immune response toward a Th1-type of immune response. Alternatively or in addition, the modified oligoribonucleotide analogs of the invention can be used in combination with an allergen to treat a subject having asthma.

The immunostimulatory composition of the invention may also be administered in conjunction with an asthma therapy. Conventional methods for treating or preventing asthma have involved the use of anti-allergy therapies (described above) and a number of other agents, including inhaled agents.

Medications for the treatment of asthma are generally separated into two categories, quick-relief medications and long-term control medications. Asthma patients take the long-term control medications on a daily basis to achieve and maintain control of persistent asthma. Long-term control medications include anti-inflammatory agents such as corticosteroids, chromolyn sodium and nedocromil; long-acting bronchodilators, such as long-acting β_2-agonists and methylxanthines; and leukotriene modifiers. The quick-relief medications include short-acting β_2 agonists, anti-cholinergics, and systemic corticosteroids. There are many side effects associated with each of these drugs and none of the drugs alone or in combination is capable of preventing or completely treating asthma.

Asthma medicaments include, but are not limited, PDE-4 inhibitors, bronchodilator/beta-2 agonists, K+ channel openers, VLA-4 antagonists, neurokin antagonists, thromboxane A2 (TXA2) synthesis inhibitors, xanthines, arachidonic acid antagonists, 5 lipoxygenase inhibitors, TXA2 receptor antagonists, TXA2 antagonists, inhibitor of 5-lipox activation proteins, and protease inhibitors.

Bronchodilator/β_2 agonists are a class of compounds which cause bronchodilation or smooth muscle relaxation. Bronchodilator/β_2 agonists include, but are not limited to, salmeterol, salbutamol, albuterol, terbutaline, D2522/formoterol, fenoterol, bitolterol, pirbuerol methylxanthines and orciprenaline. Long-acting β_2 agonists and bronchodilators are compounds which are used for long-term prevention of symptoms in addition to the anti-inflammatory therapies. Long-acting β_2 agonists include, but are not limited to, salmeterol and albuterol. These compounds are usually used in combination with corticosteroids and generally are not used without any inflammatory therapy. They have been associated with side effects such as tachycardia, skeletal muscle tremor, hypokalemia, and prolongation of QTc interval in overdose.

Methylxanthines, including for instance theophylline, have been used for long-term control and prevention of symptoms. These compounds cause bronchodilation resulting from phosphodiesterase inhibition and likely adenosine antagonism. Dose-related acute toxicities are a particular problem with these types of compounds. As a result, routine serum concentration must be monitored in order to account for the toxicity and narrow therapeutic range arising from individual differences in metabolic clearance. Side effects include tachycardia, tachyarrhythmias, nausea and vomiting, central nervous system stimulation, headache, seizures, hematemesis, hyperglycemia and hypokalemia. Short-acting β_2 agonists include, but are not limited to, albuterol, bitolterol, pirbuterol, and terbutaline. Some of the adverse effects associated with the administration of short-acting β_2 agonists include tachycardia, skeletal muscle tremor, hypokalemia, increased lactic acid, headache, and hyperglycemia.

Chromolyn sodium and nedocromil are used as long-term control medications for preventing primarily asthma symptoms arising from exercise or allergic symptoms arising from allergens. These compounds are believed to block early and late reactions to allergens by interfering with chloride channel function. They also stabilize mast cell membranes and inhibit activation and release of mediators from inosineophils and

epithelial cells. A four to six week period of administration is generally required to achieve a maximum benefit.

Anticholinergics are generally used for the relief of acute bronchospasm. These compounds are believed to function by competitive inhibition of muscarinic cholinergic receptors. Anticholinergics include, but are not limited to, ipratropium bromide. These compounds reverse only cholinerigically-mediated bronchospasm and do not modify any reaction to antigen. Side effects include drying of the mouth and respiratory secretions, increased wheezing in some individuals, and blurred vision if sprayed in the eyes.

The immunostimulatory ORN of the invention may also be useful for treating airway remodeling. Airway remodeling results from smooth muscle cell proliferation and/or submucosal thickening in the airways, and ultimately causes narrowing of the airways leading to restricted airflow. The immunostimulatory ORN of the invention may prevent further remodeling and possibly even reduce tissue build-up resulting from the remodeling process.

The immunostimulatory ORN of the invention are also useful for improving survival, differentiation, activation and maturation of dendritic cells. The immunostimulatory oligoribonucleotides have the unique capability to promote cell survival, differentiation, activation and maturation of dendritic cells.

Immunostimulatory ORN of the invention also increase natural killer cell lytic activity and antibody-dependent cellular cytotoxicity (ADCC). ADCC can be performed using an immunostimulatory ORN in combination with an antibody specific for a cellular target, such as a cancer cell. When the immunostimulatory ORN is administered to a subject in conjunction with the antibody, the subject's immune system is induced to kill the tumor cell. The antibodies useful in the ADCC procedure include antibodies which interact with a cell in the body. Many such antibodies specific for cellular targets have been described in the art and many are commercially available. In one embodiment the antibody is an IgG antibody.

In certain aspects the invention provides a method for enhancing epitope spreading. "Epitope spreading" as used herein refers to the diversification of epitope specificity from an initial focused, dominant epitope-specific immune response, directed against a self or foreign protein, to subdominant and/or cryptic epitopes on that protein

(intramolecular spreading) or other proteins (intermolecular spreading). Epitope spreading results in multiple epitope-specific immune responses.

The immune response consists of an initial magnification phase, which can either be deleterious, as in autoimmune disease, or beneficial, as in vaccinations, and a later down-regulatory phase to return the immune system to homeostasis and generate memory. Epitope spreading may be an important component of both phases. The enhancement of epitope spreading in the setting of a tumor allows the subject's immune system to determine additional target epitopes, not initially recognized by the immune system in response to an original therapeutic protocol, while reducing the possibility of escape variants in the tumor population and thus affect progression of disease.

The oligoribonucleotides of the invention may be useful for promoting epitope spreading in therapeutically beneficial indications such as cancer, viral and bacterial infections, and allergy. The method in one embodiment includes the steps of administering a vaccine that includes an antigen and an adjuvant to a subject and subsequently administering to the subject at least two doses of immunostimulatory ORN of the invention in an amount effective to induce multiple epitope-specific immune responses. The method in one embodiment includes the steps of administering a vaccine that includes a tumor antigen and an adjuvant to a subject and subsequently administering to the subject at least two doses of immunostimulatory ORN of the invention in an amount effective to induce multiple epitope-specific immune responses. The method in one embodiment involves applying a therapeutic protocol which results in immune system antigen exposure in a subject, followed by at least two administrations of an immunostimulatory oligoribonucleotide of the invention, to induce multiple epitope-specific immune responses, i.e., to promote epitope spreading. In various embodiments the therapeutic protocol is surgery, radiation, chemotherapy, other cancer medicaments, a vaccine, or a cancer vaccine.

The therapeutic protocol may be implemented in conjunction with an immunostimulant, in addition to the subsequent immunostimulant therapy. For instance, when the therapeutic protocol is a vaccine, it may be administered in conjunction with an adjuvant. The combination of the vaccine and the adjuvant may be a mixture or separate administrations, i.e., injections (i.e., same drainage field). Administration is not

necessarily simultaneous. If non-simultaneous injection is used, the timing may involve pre-injection of the adjuvant followed by the vaccine formulation.

After the therapeutic protocol is implemented, immunostimulant monotherapy begins. The optimized frequency, duration, and site of administration will depend on the target and other factors, but may for example be a monthly to bi-monthly administration for a period of six months to two years. Alternatively the administration may be on a daily, weekly, or biweekly basis, or the administration may be multiple times during a day, week or month. In some instances, the duration of administration may depend on the length of therapy, e.g., it may end after one week, one month, after one year, or after multiple years. In other instances the monotherapy may be continuous as with an intravenous drip. The immunostimulant may be administered to a drainage field common to the target.

For use in therapy, different doses may be necessary for treatment of a subject, depending on activity of the compound, manner of administration, purpose of the immunization (i.e., prophylactic or therapeutic), nature and severity of the disorder, age and body weight of the subject. The administration of a given dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units. Multiple administration of doses at specific intervals of weeks or months apart is usual for boosting antigen-specific immune responses.

Combined with the teachings provided herein, by choosing among the various active compounds and weighing factors such as potency, relative bioavailability, patient body weight, severity of adverse side-effects and preferred mode of administration, an effective prophylactic or therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the particular subject. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular therapeutic agent being administered, the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular nucleic acid and/or other therapeutic agent without necessitating undue experimentation.

Subject doses of the compounds described herein typically range from about 0.1 μg to 10,000 mg, more typically from about 1 μg/day to 8000 mg, and most typically from about 10 mg to 100 mg. Stated in terms of subject body weight, typical dosages

range from about 0.1 μg to 20 mg/kg/week (dosing typically may be given once weekly, or split into two or more doses given several days apart), more typically from about 0.1 to 10 mg/kg/week, and most typically from about 1 to 5 mg/kg/week.

The pharmaceutical compositions containing nucleic acids and/or other compounds can be administered by any suitable route for administering medications. A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular agent or agents selected, the particular condition being treated, and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed herein. For use in therapy, an effective amount of the nucleic acid and/or other therapeutic agent can be administered to a subject by any mode that delivers the agent to the desired surface, e.g., mucosal, systemic.

Administering the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan. Routes of administration include but are not limited to oral, parenteral, intravenous, intramuscular, intraperitoneal, intranasal, sublingual, intratracheal, inhalation, subcutaneous, ocular, vaginal, and rectal. For the treatment or prevention of asthma or allergy, such compounds are preferably inhaled, ingested or administered by systemic routes. Systemic routes include oral and parenteral. Inhaled medications are preferred in some embodiments because of the direct delivery to the lung, the site of inflammation, primarily in asthmatic patients. Several types of devices are regularly used for administration by inhalation. These types of devices include metered dose inhalers (MDI), breath-actuated MDI, dry powder inhaler (DPI), spacer/holding chambers in combination with MDI, and nebulizers.

The therapeutic agents of the invention may be delivered to a particular tissue, cell type, or to the immune system, or both, with the aid of a vector. In its broadest sense, a vector is any vehicle capable of facilitating the transfer of the compositions to the target cells. The vector generally transports the immunostimulatory nucleic acid, antibody, antigen, and/or disorder-specific medicament to the target cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector.

In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological vectors and chemical/physical vectors are useful in the delivery and/or uptake of therapeutic agents of the invention.

Most biological vectors are used for delivery of nucleic acids and this would be most appropriate in the delivery of therapeutic agents that are or that include immunostimulatory nucleic acids.

In addition to the biological vectors discussed herein, chemical/physical vectors may be used to deliver therapeutic agents including immunostimulatory nucleic acids, antibodies, antigens, and disorder-specific medicaments. As used herein, a chemical/physical vector refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the nucleic acid and/or other medicament.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vessels which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUVs), which range in size from 0.2 - 4.0 μm can encapsulate large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form. Fraley et al. (1981) *Trends Biochem Sci* 6:77.

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to an immune cell include, but are not limited to: intact or fragments of molecules which interact with immune cell specific receptors and molecules, such as antibodies, which interact with the cell surface markers of immune cells. Such ligands may easily be identified by binding assays well known to those of skill in the art. In still other embodiments, the liposome may be targeted to the cancer by coupling it to a one of the immunotherapeutic antibodies discussed earlier. Additionally, the vector may be coupled to a nuclear targeting peptide, which will direct the vector to the nucleus of the host cell.

Lipid formulations for transfection are commercially available from QIAGEN, for example, as EFFECTENE™ (a non-liposomal lipid with a special DNA condensing enhancer) and SUPERFECT™ (a novel acting dendrimeric technology).

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis G (1985) *Trends Biotechnol* 3:235-241.

Certain cationic lipids, including in particular N-[1-(2, 3 dioleoyloxy)-propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP), appear to be especially advantageous when combined with the modified oligoribonucleotide analogs of the invention.

In one embodiment, the vehicle is a biocompatible microparticle or implant that is suitable for implantation or administration to the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO95/24929, entitled "Polymeric Gene Delivery System". PCT/US/0307 describes a biocompatible, preferably biodegradable polymeric matrix for containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix can be used to achieve sustained release of the therapeutic agent in the subject.

The polymeric matrix preferably is in the form of a microparticle such as a microsphere (wherein the nucleic acid and/or the other therapeutic agent is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein the nucleic acid and/or the other therapeutic agent is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the therapeutic agent include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix is introduced. The size of the polymeric matrix further is selected according to the method of delivery which is to be used, typically injection into a tissue or administration of a suspension by aerosol into the nasal and/or pulmonary areas. Preferably when an aerosol route is used the polymeric matrix and the nucleic acid and/or the other therapeutic agent

are encompassed in a surfactant vehicle. The polymeric matrix composition can be selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the matrix is administered to a nasal and/or pulmonary surface that has sustained an injury. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time. In some preferred embodiments, the nucleic acid are administered to the subject via an implant while the other therapeutic agent is administered acutely. Biocompatible microspheres that are suitable for delivery, such as oral or mucosal delivery, are disclosed in Chickering et al. (1996) *Biotech Bioeng* 52:96-101 and Mathiowitz E et al. (1997) *Nature* 386:410-414 and PCT Pat. Application WO97/03702.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver the nucleic acid and/or the other therapeutic agent to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable, particularly for the nucleic acid agents. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H.S. Sawhney, C.P. Pathak and J.A. Hubell in *Macromolecules*, (1993) 26:581-587, the teachings of which are incorporated herein. These include polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate).

If the therapeutic agent is a nucleic acid, the use of compaction agents may also be desirable. Compaction agents also can be used alone, or in combination with, a biological or chemical/physical vector. A "compaction agent", as used herein, refers to

an agent, such as a histone, that neutralizes the negative charges on the nucleic acid and thereby permits compaction of the nucleic acid into a fine granule. Compaction of the nucleic acid facilitates the uptake of the nucleic acid by the target cell. The compaction agents can be used alone, i.e., to deliver a nucleic acid in a form that is more efficiently taken up by the cell or, more preferably, in combination with one or more of the above-described vectors.

Other exemplary compositions that can be used to facilitate uptake of a nucleic acid include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a nucleic acid into a preselected location within the target cell chromosome).

The compounds may be administered alone (e.g., in saline or buffer) or using any delivery vehicle known in the art. For instance the following delivery vehicles have been described: cochleates; Emulsomes®; ISCOM®s; live bacterial vectors (e.g., *Salmonella, Escherichia coli, Bacillus Calmette-Guérin, Shigella, Lactobacillus*); live viral vectors (e.g., Vaccinia, adenovirus, Herpes Simplex); microspheres; nucleic acid vaccines; polymers (e.g. carboxymethylcellulose, chitosan); polymer rings; proteosomes; sodium fluoride; transgenic plants. In some embodiments of the invention the delivery vehicle is a liposome, a pH-sensitive liposome (e.g. OctoPlus®), a fusiogenic liposome, a niosome, a lipoplexe, a polyplexe, a lipopolyplexe, a water-in-oil (W/O) emulsion, an oil-in-water (O/W) emulsion, a water-in-oil-in water (W/O/W) multiple emulsion, a micro-emulsion, a nano-emulsion, a micelle , a dendrimer, a virosome, a virus-like particle, a polymeric nanoparticle, as a nanosphere or a nanocapsule, a polymeric microparticle, as a microsphere or a microcapsule. The formulations of the invention are administered in pharmaceutically acceptable solutions, which may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, adjuvants, and optionally other therapeutic ingredients. In some embodiments the composition is sterile.

The term pharmaceutically-acceptable carrier means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term carrier denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is

combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

For oral administration, the compounds (i.e., nucleic acids, antigens, antibodies, and other therapeutic agents) can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. Pharmaceutical preparations for oral use can be obtained as solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Optionally the oral formulations may also be formulated in saline or buffers for neutralizing internal acid conditions or may be administered without any carriers.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid

paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. Microspheres formulated for oral administration may also be used. Such microspheres have been well defined in the art. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds, when it is desirable to deliver them systemically, may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active compounds may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long-acting formulations may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Suitable liquid or solid pharmaceutical preparation forms are, for example, aqueous or saline solutions for inhalation, microencapsulated, encochleated, coated onto microscopic gold particles, contained in liposomes, nebulized, aerosols, pellets for implantation into the skin, or dried onto a sharp object to be scratched into the skin. The pharmaceutical compositions also include granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, drops or preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders, coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of methods for drug delivery, see Langer R (1990) *Science* 249:1527-1533, which is incorporated herein by reference.

The nucleic acids and optionally other therapeutics and/or antigens may be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts can be

prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group.

Suitable buffering agents include: acetic acid and a salt (1-2% w/v); citric acid and a salt (1-3% w/v); boric acid and a salt (0.5-2.5% w/v); and phosphoric acid and a salt (0.8-2% w/v). Suitable preservatives include benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product. Liquid dose units are vials or ampoules. Solid dose units are tablets, capsules and suppositories.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the compounds, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di-, and tri-glycerides; hydrogel release systems; silastic systems; peptide-based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which an agent of the invention is contained in a form within a matrix such as those described in U.S. Pat. Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Pat. Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

The present invention is further illustrated by the following Examples, which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Materials and Methods:

Oligoribonucleotides

All ORN were purchased from Biospring (Frankfurt, Germany) or provided by Coley Pharmaceutical GmbH (Duesseldorf, Germany), controlled for identity and purity by Coley Pharmaceutical GmbH and had undetectable endotoxin levels (<0.1EU/ml) measured by the Limulus assay (BioWhittaker, Verviers, Belgium). ORN were suspended in sterile, DNAse- and RNAse-free dH_2O (Life Technologies, Eggenstein, Germany) and stored and handled under aseptic conditions to prevent both microbial and endotoxin contamination. All dilutions were carried out using endotoxin-free Tris-EDTA or DNAse- and RNAse-free dH_2O. ORN sequences are provided in Table 1 below.

Cell purification

Examples 1-7 and 10: Peripheral blood buffy coat preparations from healthy human donors were obtained from the Blood Bank of the University of Düsseldorf (Germany) and PBMC were purified by centrifugation over Ficoll-Hypaque (Sigma). Cells were cultured in a humidified incubator at 37°C in RPMI 1640 medium supplemented with 5% (v/v) heat inactivated human AB serum (BioWhittaker) or 10% (v/v) heat inactivated FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (all from Sigma). PDCs and monocytes were isolated with the BDCA-4 pDC or CD14 monocyte isolation kit (Miltenyi).

Example 8: Spleens were harvested from naïve sv129 mice. Dendritic cells (DC) were purified with magnetic beads (CD11c+) from Myltenyi. Cells were cultured in a humidified incubator at 37°C for 20 hours.

Cytokine detection

Examples 1-6 and 10: PBMC were resuspended at a concentration of about $5x10^6$ cells/ml and added to 96 well round-bottomed plates (250µl/well). PBMC were incubated with various ORN concentrations (in the presence of DOTAP: 50 mg/ml with 2 µM of ORN) and culture supernatants (SN) were collected 24 hours later. If not used immediately, SN were stored at –20°C until required.

Example 7: Human PBMC (n=2), monocytes or pDC were stimulated with SEQ ID NO 14 (4µM), SEQ ID NO:28 (0.5µM), or SEQ ID NO 12 (0.5µM) in the presence of DOTAP (20µg/ml] for 24 hours and IFN-α measured.

Example 8: In a 96 well plate, purified DC were seeded at 3 x 10^5 cells per well and activated with 5µM, 1µM, 200nM, 40nM, 8nM or 1.6nM of specified reagents formulated with DOTAP in a 200µL volume. Cells were incubated in a humidified incubator at 37°C for 20 hours and SN collected.

Amounts of cytokines (IFN-α, IFN-γ or IL-12p40) in the SN were assessed using a commercially available ELISA Kit for IL-12p40 (from BD Biosciences, Heidelberg, Germany), IFN-γ (from Diaclone, Besançon, France) or an in-house ELISA for IFN-α developed using commercially available antibodies (PBL, New Brunswick, NJ, USA). For analysis of a broad set of cytokines and chemokines, multiplex analysis with a luminex system from Bio-Rad (Munich, Germany) and Multiplex kits from Biosource (Solingen, Germany) was performed.

In vivo cytokine induction

sv129 mice (3 per group) were injected intravenously with ORN or small molecule formulated with DOTAP at a 2:1 (w/w) ratio. Plasma was harvested 3 hours post injection and ELISA assay performed.

Introduction

The instant invention in some aspects involves the discovery that certain sequence-specific RNA motifs are immunostimulatory, acting through TLR7, as opposed

to other motifs that act on TLR7 and TLR8 (GU rich and CU rich motifs lacking poly-G ends) or on TLR8 only (AU rich motifs lacking poly-G ends). ORN preferably containing an immunostimulatory ORN motif directly or indirectly flanked by poly G motif(s) stimulate an immune response through TLR7 and not TLR8. Differences between IFN-α, TNF-α, IFN-γ and IL-12 production have been observed in these distinct classes of ORN, e.g. ORN containing AU- and GU-containing repetitions but lacking poly-G ends and the ORN of the invention. Interestingly the immunostimulatory ORN of the invention have been found to produce a strong IFN-α response but do not stimulate other typical cytokines, for instance those induced in response to TLR8 stimulation. Quite surprisingly it was also discovered according to the invention that immunostimulatory ORN motifs such as those which mediate TLR7/8 and TLR8 responses produce a TLR7 immune profile when one or more poly-G motifs is incorporated into the ORN.

Example 1:ORN of the invention induce high amounts of IFN-α but not IL-12p40

To test the immune stimulatory capacity of the ORN of the invention, PBMC were incubated with ORN in the presence of DOTAP. After 24 hours supernatants were assayed for the presence of IFN-α (Figure 1A) or IL-12p40 (Figure 1B). The ORN of the invention, SEQ ID NO:4, SEQ ID NO:8 and SEQ ID NO:5 (Table1), induced very high amounts of the TLR7-associated cytokine IFN-α but no substantial induction of the TLR8-associated cytokine IL-12-p40. In contrast, control SEQ ID NO:1 and 2, known activators of TLR7 and TLR8-associated responses, induced both high amounts of IFN-α and high amounts of IL-12p40.

Example 2: U-rich sequences are necessary for cytokine production

To test the sequence requirements of the region of the ORN flanked by the poly G regions, ORN containing U-rich sequences (with either a phosphodiester (PO) or phosphorothioate (PS) backbone) embedded within oligo(rG) stretches at the 5' and 3' end were tested for their ability to induce cytokine production (Figure 2). The ORN with the U-rich sequence flanked by G stretches, SEQ ID NO:6, induced high amounts of IFN-α (Figure 2A) but no substantial amount of IL-12p40 (Figure 2B). The ORN without U within their sequence, SEQ ID NO:3 and 7, did not induce cytokine production.

Example 3: ORN with oligo(dG) stretches at the 3' end induce TLR7-associated cytokines

To determine whether the 5' oligo(rG) stretch was necessary for inducing a TLR7-like immune response, an ORN with an oligo(rA) 3' end (SEQ ID NO:9), an oligo(dG) 3' end (SEQ ID NO:10), a 3' cholesterol tag (SEQ ID NO:11) and no tail (SEQ ID NO:12) were tested for their ability to induce TLR7-like and TLR8-like cytokine responses. As shown in Figure 3, the ORN containing only a 3' oligo(dG) (SEQ ID NO:10) stretch induced a TLR7-like immune response. SEQ ID NO:10 induced high amounts of IFN-α (Figure 3A) but not IFN-γ (Figure 3B). The positive control (SEQ ID NO:1) and SEQ ID NO:9 induced TLR7/8-like immune responses, whereas SEQ ID NO:11 and 12 induced TLR8-like immune responses.

Example 4: Efficient IFN-α induction is correlated with the formation of tertiary structures.

It is known that (G)n stretches in oligonucleotides, where $n \geq 4$, leads to intermolecular aggregate formation. The uptake of oligonucleotides with (G)n stretches is about 20 to 40-times higher than of non-aggregated oligonucleotides and the intracellular localization appears also to be different. It is not understood how these observations correlate with biological activity. In order to determine whether aggregate formation played a role in the activation of TLR7-like immune responses by the ORN, Human PBMC were incubated with SEQ ID NO:10 and 13 in the absence or presence of DOTAP and IFN-α was measured. SEQ ID NO:13 is the same sequence as SEQ ID NO:10 but with one 7-deaza-rG interrupting the poly dG stretch. Both in the presence and absence of uptake enhancer the modified ORN resulted in reduced cytokine production. The induction of IFN-α by SEQ ID NO:10 was much stronger than the modified SEQ ID NO:13, suggesting that aggregate formation plays a role in activation of TLR7-induced IFN-α production.

Example 5: Oligo(rG) and oligo(dG) at the 3' end are sufficient to induce a TLR7-like immune response

In order to compare the ability of an ORN to induce IFN-α when modified with various 3' tags, the activity of SEQ ID NO:12 was compared to that of the same sequence

with a 3' poly rA stretch (SEQ ID NO:9), a 3' poly dG stretch (SEQ ID NO:10), a 3' cholesterol tag (SEQ ID NO:11), a 3' triethylene glycol (SEQ ID NO:14), a 3' acridine tag (SEQ ID NO:16), a 3' fluorescein tag (SEQ ID NO:17), a 3' biotin tag (SEQ ID NO:18), a 3' hexadecylglycerol tag (SEQ ID NO:20), and a 3' poly rG stretch (SEQ ID NO:21). Human PBMC were incubated with the indicated ORNs in the absence of DOTAP for 24h and IFN-α was measured. Of the 3' modifications used only ORN with oligo(rG) and oligo(dG) and to some extent poly rA led to IFN-α production in the absence of DOTAP.

Example 6: The number of G residues determines the increase in IFN-α versus decrease of TNF-α and other cytokine production.

In order to compare the ability of an ORN to induce IFN-α and TNF-α with a varying number of 3' G residues, the activity of SEQ ID NO:21 was compared to that of the same sequence with the addition of one 3' rG (SEQ ID NO:23), the deletion of one 3' rG (SEQ ID NO:24), and the deletion of 3 3' rG (SEQ ID NO:25) (Figure 6). Also tested were an ORN with an immunostimulatory motif (UUGU) with a 3' poly rG stretch (SEQ ID NO:26), and a UUUU motif with a 3' poly rG stretch (SEQ ID NO:27).

A decrease of the 3' Gs lead to a reduction of the IFN-α levels to levels only slightly enhanced when compared to the unmodified ORN SEQ ID NO:1. In addition, the decrease of the poly G tail also led to a expression of the TLR8 associated cytokine TNF-α, and this effect was dependent on the number of poly rGs. A minimum of 2 Gs appears to be sufficient to increase IFN-α and decrease other effects.

Example 7: ORN with poly rG stimulate TLR7-dependent IFN-α production in pDC that lack TLR8

In order to demonstrate that ORN with 3' poly rG stretches are acting through TLR7, Human PBMC, monocytes or pDC were stimulated with SEQ ID NO 14 (4μM), SEQ ID NO:28 (0.5μM), or SEQ ID NO 12 (0.5μM) in the presence of DOTAP (20μg/ml) for 24h and IFN-α was measured. ORN and CpG ODN were found to stimulate IFN-α from PBMC. This response was greatly reduced in monocytes that express minimal, if at all, levels of TLR7. However, strong IFN-α production was observed from TLR7-expressing pDC, although the levels were somewhat lower than the

CpG ODN. The levels were, however, nevertheless about 5 times higher than those observed in PBMC.

Example 8: Stimulation of cytokine production in murine dentritic cells

The ORN of the invention were tested for their ability to induce cytokine production in murine dendritic cells (DC). Murine CD11+ splenocytes were harvested and treated with ORN for 20 hours. Supernatants were analyzed by ELISA for IFN-α (Figure 8A), IL-6 (Figure 8B), IL-12p40 (Figure 8C) and IP-10 (Figure 8D) concentration. Cells were treated with a know TLR 7/8 stimulatory ORN (SEQ ID NO:48) with DOTAP (DO), the small molecule R-848 which stimulates TLR7/8, the cholesterol tagged and 3'G stretch modified ORN of SEQ ID NO:12 (SEQ ID NO:11 and 12, respectively, both with and without DOTAP), and SEQ ID NO:12 with DOTAP. SEQ ID NO:21 and 48, both formulated with DOTAP, induced high amounts of IFN-α . Unformulated SEQ ID NO:21 induced a high amount of IFN-α at a slightly higher dose. When formulated with DOTAP, both SEQ ID NO:21 AND SEQ ID NO:48 induced IL-12p40, IL-6, and IP-10. This was expected, as mice do not have a known functional TLR8 equivalent to human TLR8, the cytokine profile induced from murine TLR7 alone is similar to that induced by TLR7/8 activators in humans.

Example 9: In vivo *cytokine induction*

The ORN of the invention were tested for their ability to induce cytokine production *in vivo* (Figure 9). sv129 mice were injected intravenously with an unmodified ORN (SEQ ID NO:12), a cholesterol modified ORN of the same sequence (SEQ ID NO:11), an ORN with the same sequence and a 3' poly G stretch (SEQ ID NO:21), or R-848. All ORN were formulated with DOTAP at a 2:1 ratio (w/w). Mice were bled and serum analyzed for IFN-α (Figure 9A), IL-12p40 (Figure 9B), IP-10 (Figure 9C) and TNF-α (Figure 9D) concentration by ELISA. SEQ ID NO:21 induced IFN-α to a greater extent than the cholesterol modified or unmodified ORN. At a higher dose, SEQ ID NO:21 induce IP-10 and induced IFN-α to a greater degree that R-848 (either alone or with DOTAP). SEQ ID NO:21 did not induce substantial amounts of IL-12p40 or TNF-α in this assay as the response *in vivo* was slightly weaker than the response *in vitro* (Figure 10). Serum was tested 3 hours after injection for IP-10 concentration. As shown in

Figure 10A, SEQ ID NO:21 formulated with DOTAP induced IP-10 to a greater extent than SEQ ID NO:21 alone. However, as shown in figure 10B, SEQ ID NO:21 induced slightly less IP-10 than thecholesterol modified ORN SEQ ID NO:11.

Example 10: 3' polyG modified ORN stimulate TLR7 in the absence of formulation

In order to demonstrate that 3' polyG modified ORN stimulate TLR7 in the absence of formulation, 3' variations were made to the base sequence of SEQ ID NO:48, wherein 5 bases were removed from the 3' end and replaces with 5 dG residues (SEQ ID NO:42), 4 dG residues and a 3-methyl-dG (SEQ ID NO:43), 5 dG residues and a dT (SEQ ID NO:44), 5 dG residues and an inverted dT(SEQ ID NO:45), 4 dG residues and a 3-methyl-dG with phosphodiester internucleotide linkages replacing the phosphorothioate linkages (SEQ ID NO:46), or 4 dG residues and an rG residue (SEQ ID NO:47). As shown in Figure 11, all unformulated modified poly-G ORN induced IFN-α to a greater degree than SEQ ID NO:48, although not to the degree of SEQ ID NO:48 + DOTAP.

Table 1: ORN and ODN sequences

SEQ ID #	SEQUENCE
1	rC*rC*rG*rU*rC*rU*rG*rU*rU*rG*rU*rG*rU*rG*rA*rC*rU*rC
2	rU*rU*rU*rU*rU*rU*rU
3	rG*rG*rG*rG*rA*rA*rA*rA*rA*rA*rA*rA*rA*rArG*rG*rG*rG*rG*rG*rG
4	rG*rG*rG*rG*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rG*rG*rG*rG*rG*rG*rG
5	rG*rG*rG*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rG*rG*rG*rG*rG*rG*rG
6	rG*rG*rG*rGr*U*rU*rA*rU*rU*rA*rU*rU*rA*rU*rG*rG*rG*rG*rG*rG*rG
7	rG*rG*rG*rG-rA-rA-rA-rA-rA-rA-rA-rA-rA-rA-rG*rG*rG*rG*rG*rG*rG
8	rG*rG*rG*rG-rU-rU-rU-rU-rU-rU-rU-rU-rU-rU-rG*rG*rG*rG*rG*rG*rG
9	rG*rU*rU*rG*rU*rG*rU*rA*rA*rA*rA*rA
10	rG*rU*rU*rG*rU*rG*rU*dG*dG*dG*dG*dG
11	rG*rU*rU*rG*rU*rG*rU-chol
12	rG*rU*rU*rG*rU*rG*rU
13	rG*rU*rU*rG*rU*rG*rU*G*E*G*G*G
14	rG*rU*rU*rG*rU*rG*rU-teg
16	rG*rU*rU*rG*rU*rG*rU-acr
17	rG*rU*rU*rG*rU*rG*rU-fam
18	rG*rU*rU*rG*rU*rG*rU-biot
20	rG*rU*rU*rG*rU*rG*rU-hex
21	rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG*rG
22	chol-rG*rU*rU*rG*rU*rG*rU
23	rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG*rG*rG
24	rG*rU*rU*rG*rU*rG*rU*rG*rG*rG*rG
25	rG*rU*rU*rG*rU*rG*rU*rG*rG
26	rU*rU*rG*rU*rG*rG*rG*rG*rG

27	rU*rU*rU*rU*rG*rG*rG*rG*rG
28	dT*dC*dG*dT*dC*dG*dT*dT*dT*dT*dC*dG*dG*dC*dG*dC*dG*dC*dG*dC*dC*dG
42	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*G*G*G*G*G
43	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*G*G*G*G*3mG
44	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*G*G*G*G*G*T
45	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*G*G*G*G*G-iT
46	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU-G-G-G-G-3mG
47	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*G*G*G*G*rG
48	rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rU

*	phosphorothioate backbone
-	phosphodiester backbone
chol	cholesterol
E	7-deaza-rG
teg	triethylene glycol
acr	acridine
fam	florescein
biot	biotin
hex	hexadecylglycerol
rN	ribonucleotide
dN	deoxyribonucleotide
3mG	3 methylguanosine
iN	inverted nucleotide

Having thus described several aspects of at least one embodiment of this invention, it is to be appreciated various alterations, modifications, and improvements will readily occur to those skilled in the art. Such alterations, modifications, and improvements are intended to be part of this disclosure, and are intended to be within the spirit and scope of the invention. Accordingly, the foregoing description and drawings are by way of example only.

What is claimed is:

CLAIMS

1. An RNA oligonucleotide (ORN) of 10-100 ribonucleotides in length comprising: $GG(R_1)_n\ (U)_{4\text{-}20}(R_2)_m$ GGGG (SEQ ID NO:29) wherein R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker, U is Uridine or a derivative thereof, G is guanosine, wherein n=0-20, wherein m= 0-20.

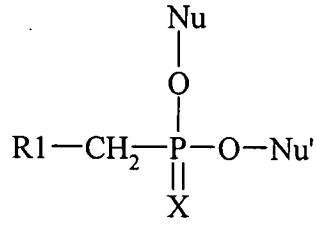
2. The ORN of claim 1, wherein the ORN is not 5' GGGGUUUUGGGGG 3' (SEQ ID NO. 33) or 5' GGGGUUUUGGGG 3'(SEQ ID NO. 34).

3. An RNA oligonucleotide (ORN) of 10-100 ribonucleotides in length comprising: $GG(R_1)_n\ (U)_{5\text{-}20}(R_2)_m$ GGGG (SEQ ID NO:30) wherein R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker, U is Uridine or a derivative thereof, G is guanosine, wherein n=0-20, wherein m= 0-20.

4. An RNA oligonucleotide (ORN) of 10-100 ribonucleotides in length comprising: $GG(R_1)_n\ (U)_4(R_2)_m$ GGGG (SEQ ID NO:31) wherein R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker, U is Uridine or a derivative thereof, G is guanosine, wherein n=0-20, wherein m= 0-20 and wherein when $(R_1)_n$ is GG $(R_2)_m$ is not G or m is not = 0.

5. An RNA oligonucleotide (ORN) of 10-100 ribonucleotides in length comprising: $GG(R_1)_n\ (U)_{4\text{-}20}(R_2)_m$ GGGG (SEQ ID NO:29) wherein R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker, U is Uridine or a derivative thereof, G is guanosine, wherein n=0-20, wherein m= 0-20, and wherein the ORN does not include a modified phosphate linkage selected from the group consisting of:

(i)



Formula I

wherein

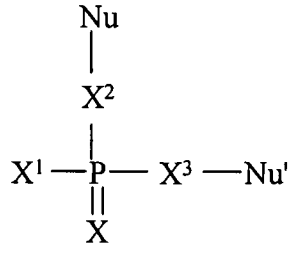
R1 is hydrogen (H), COOR, OH, C1-C18 alkyl, C_6H_5, or $(CH_2)_m$-NH-R2, wherein R is H or methyl, butyl, methoxyethyl, pivaloyl oxymethyl, pivaloyl oxybenzyl, or S-pivaloyl thioethyl; R2 is H, C1-C18 alkyl, or C2-C18 acyl; and m is 1 to 17;

X is oxygen (O) or sulfur (S); and

each of Nu and Nu' independently is a nucleoside or nucleoside analog;

with the proviso that if R1 is H, then X is S;

(ii)



Formula II

wherein

X is O or S;

X^1 is OH, SH, BH_3, OR3, or NHR3, wherein R3 is C1-C18 alkyl;

each of X^2 and X^3 independently is O, S, CH_2, or CF_2; and

each of Nu and Nu' independently is a nucleoside or nucleoside analog;

with the proviso that

(a) at least one of X, X^2, and X^3 is not O or X^1 is not OH,

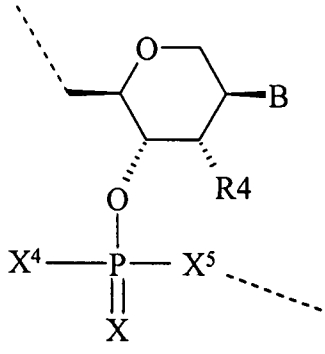
(b) if X^1 is SH, then at least one of X, X^2, and X^3 is not O,

(c) if X and X^2 are O and if X^1 is OH, then X^3 is not S and Nu is 3'Nu and Nu' is 5'Nu', and

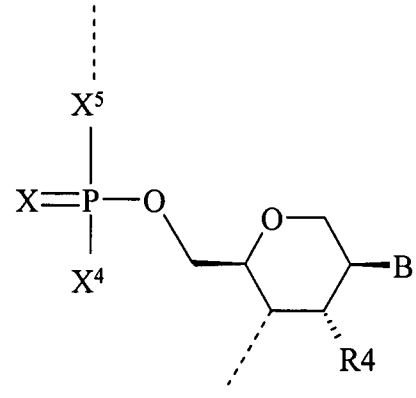
(d) if X^1 is BH_3, then at least one of X, X^2, or X^3 is S; and

(iii) any combination of (i) and (ii)

or at least one nucleotide analog provided as Formula IIIA or Formula IIIB



Formula IIIA



Formula IIIB

wherein

R4 is H or OR, wherein R is H or C1-C18 acyl;

B is a nucleobase, a modified nucleobase, or H;

each of X and X^5 independently is O or S; and

X^4 is OH, SH, methyl, or NHR5, wherein R5 is C1-C18 alkyl; and

each dashed line independently represents an optional bond to an adjacent unit, hydrogen, or an organic radical;

with the proviso that at least one of X and X^5 is not O or X^4 is not OH.

6. The ORN of claim 1, wherein the ORN does not include a 5' GGGUUUU 3' motif.

7. The ORN of claim 1, further comprising a sterile carrier.

8. The ORN of claim 1, wherein the ORN is formulated with a lipid carrier.

9. The ORN of claim 1, wherein the ORN is single stranded.

10. The ORN of claim 1, wherein the oligonucleotide is not an siRNA or antisense oligonucleotide.

11. The ORN of claim 1, wherein the ORN includes at least one phosphorothioate linkage.

12. The ORN of claim 1, wherein all internucleotide linkages of the ORN are phosphorothioate linkages.

13. The ORN of claim 1, wherein the ORN includes at least one phosphodiester-like linkage.

14. The ORN of claim 13, wherein the phosphodiester-like linkage is a phosphodiester linkage.

15. The ORN of claim 1, further comprising at least one 5'-5' internucleotide linkage.

16. The ORN of claim 15, wherein the 5'-5' internucleotide linkage comprises a linker.

17. The ORN of claim 1, further comprising at least one 3'-3' internucleotide linkage.

18. The ORN of claim 17, wherein the 3'-3' internucleotide linkage comprises a linker.

19. The ORN of claim 1, further comprising at least one 2'-O-alkyl-modified, 2-fluoro-arabino-modified, or LNA-modified G.

20. The ORN of claim 1, wherein the ORN does not include a CG dinucleotide.

21. The ORN of claim 1, wherein the ORN includes at least one unmethylated CpG dinucleotide.

22. The ORN of claim 1, wherein the ORN comprises a sequence of nucleosides, nucleoside analogs, or a combination of nucleosides and nucleoside analogs capable of forming secondary structure provided by at least two adjacent hydrogen-bonded base pairs.

23. The ORN of claim 22, wherein the secondary structure is a stem-loop secondary structure.

24. The ORN of claim 1, wherein $(R_1)_n$ is GG.

25. The ORN of claim 1, wherein $(R_2)_m$ is GGG

26. The ORN of claim 1, wherein $(U)_{4-20}$ is UUUUU.

27. The ORN of claim 1, wherein $(U)_{4-20}$ is UUUUUUU.

28. The ORN of claim 1, wherein $(U)_{4-20}$ is UUUUUUUUUU (SEQ ID NO:32).

29. The ORN of claim 1, wherein GG and $(R_1)_n$ are connected directly.

30. The ORN of claim 1, wherein GG and $(R_1)_n$ are connected via a 3'-3' linkage.

31. The ORN of claim 1, wherein GG and $(R_1)_n$ are connected by a spacer.

32. The ORN of claim 31, wherein the spacer is a non-nucleotide spacer.

33. The ORN of claim 32, wherein the non-nucleotide spacer is a D-spacer.

34. The ORN of claim 32, wherein the non-nucleotide spacer is a linker.

35. An ORN comprising rG*rG*rG*rG*rU*rU*rU*rU*rU*rU*rU*rU*rU*rU*rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:4).

36. An ORN comprising rG*rG*rG*rG*rU*rU*rG*rU*rU*rG*rU*rU*rG*rU*rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:5).

37. An ORN comprising rG*rG*rG*rG*rU*rU*rA*rU*rU*rA*rU*rU*rA*rU*rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:6).

38. An ORN comprising rG*rG*rG*rG-rU-rU-rU-rU-rU-rU-rU-rU-rU-rU-rG*rG*rG*rG*rG*rG*rG (SEQ ID NO:8).

39. An ORN comprising rG*rU*rU*rG*rU*rG*rU*dG*dG*dG*dG*dG (SEQ ID NO:10).

40. An immunostimulatory RNA oligonucleotide (ORN) of 8-100 ribonucleotides in length comprising: an immunostimulatory ORN motif linked to a poly-G motif, wherein the poly-G motif is 3' to the immunostimulatory ORN motif and the poly-G motif comprises at least 4 Gs, wherein G is guanosine.

41. The ORN of claim 40, wherein the ORN is not one of the following 5' GGGGUUUUGGGGG 3' (SEQ ID NO:33), 5' GGGGUUUUGGGG 3' (SEQ ID NO:34), GUUUUUG (SEQ ID NO 35), GGGGGGGUUGUGUGGGGG (SEQ ID NO:36), CCCCUUUUGGGGG (SEQ ID NO:37), GUUUGUGUGGGG (SEQ ID NO:38), GUUGUGUGGGGG (SEQ ID NO:39), UUUUUUGGGGG (SEQ ID NO:40), UUUUUGGGGG (SEQ ID NO:41), UUUUGGGGG (SEQ ID NO:19), or UUUUGGGG (SEQ ID NO:15).

42. The ORN of claim 40, wherein the immunostimulatory ORN motif is a TLR8 motif.

43. The ORN of claim 42, wherein the TLR8 motif is N-U-R_1-R_2, wherein N is a ribonucleotide and N does not include a U, U is Uracil or a derivative thereof and wherein R is a ribonucleotide wherein at least one of R_1 and R_2 is Adenosine (A) or Cytosine or derivatives thereof. R is not U unless N-U-R_1-R_2 includes at least two A.

44. The ORN of claim 43, wherein N is Adenosine or Cytosine (C) or derivatives thereof.

45. The ORN of claim 43, further comprising a second N-U-R_1-R_2 motif.

46. The ORN of claim 40, wherein the immunostimulatory ORN motif is a TLR7/8 motif.

47. The ORN of claim 46, wherein the TLR7/8 motif comprises a ribonucleotide sequence selected from the group consisting of:

(i) 5'-C/U-U-G/U-U-3',

(ii) 5'-R-U-R-G-Y-3',

(iii) 5'-G-U-U-G-B-3',

(iv) 5'-G-U-G-U-G/U-3', and

(v) 5'-G/C-U-A/C-G-G-C-A-C-3',

wherein C/U is cytosine (C) or uracil (U), G/U is guanine (G) or U, R is purine, Y is pyrimidine, B is U, G, or C, G/C is G or C, and A/C is adenine (A) or C.

48. The ORN of claim 40, wherein the poly G motif is 6 G's

49. The ORN of claim 40, wherein the poly G motif is 7 G's.

50. The ORN of claim 40, wherein the immunostimulatory ORN motif and the poly-G motif are directly linked.

51. The ORN of claim 40, wherein the immunostimulatory ORN motif and the poly-G motif are indirectly linked by a linker that is a spacer, a nucleotidic linker or a non-nucleotidic linker.

52. The ORN of claim 40, wherein the ORN includes at least one phosphorothioate linkage.

53. The ORN of claim 40, wherein all internucleotide linkages of the ORN are phosphorothioate linkages.

54. The ORN of claim 52, wherein the ORN includes at least one phosphodiester-like linkage.

55. The ORN of any one of claims 1, 3, 4, 5, or 35-40 wherein the ORN is conjugated to a molecule chosen from the group consisting of a lipid, a small molecule, a peptide, or a protein.

56. The ORN of claim 55 wherein the ORN and the molecule are conjugated directly.

57. The ORN of claim 55 wherein the ORN and the molecule are conjugated by means of a linker.

58. A method for stimulating production of IFN-α, comprising:

contacting a TLR7 expressing cell with an RNA oligonucleotide (ORN) comprising: an immunostimulatory ORN motif linked to a poly-G motif, wherein the poly-G motif is 3' to the immunostimulatory ORN motif and the poly-G motif comprises at least 4 Gs, wherein G is guanosine, in an effective amount to stimulate IFN-α production and wherein IFN-γ or IL-12 production in response to the ORN is not induced significantly relative to background.

59. The method of claim 57, wherein the ORN is $GG(R_1)_n\ (U)_{4-20}(R_2)_m\ GGGG$ wherein R_1 and R_2 are a ribonucleoside, a deoxyribonucleoside, a spacer, or a non-nucleotidic linker, wherein n=0-20, wherein m= 0-20, U is Uridine or a derivative thereof, G is guanosine.

60. The method of claim 58, wherein the TLR7 expressing cell is a mDC.

61. The method of claim 58, wherein the TLR7 expressing cell is *in vitro*.

62. The method of claim 58, wherein the TLR7 expressing cell is *in vivo*.

63. A method for treating cancer comprising;

administering to a subject in need thereof an ORN of any one of claims 1-57 in an effective amount to treat the cancer.

64. The method of claim 63, further comprising administering a chemotherapeutic to the subject.

65. The method of claim 64, further comprising administering radiation to the subject.

66. A method for treating asthma, comprising administering to a subject in need thereof an ORN of any one of claims 1-57 in an effective amount to treat asthma.

67. A method for treating allergy, comprising administering to a subject in need thereof an ORN of any one of claims 1-57 in an effective amount to treat allergy.

68. The method of claim 67, wherein the subject has allergic rhinitis.

69. A method for modulating an immune response in a subject, comprising administering to a subject in need thereof an ORN of any one of claims 1-57 in an effective amount to modulate an immune response.

70. The method of claim 69, wherein the ORN is delivered to the subject to treat autoimmune disease in the subject.

71. The method of claim 69, wherein the ORN is delivered to the subject to treat airway remodeling in the subject.

72. The method of claim 69, wherein the ORN is administered without an antigen to the subject.

73. The method of claim 69, wherein the ORN is delivered by a route selected from the group consisting of oral, nasal, sublingual, intravenous, subcutaneous, mucosal, respiratory, direct injection, and dermally.

74. The method of claim 69, wherein the ORN is delivered to the subject in an effective amount to induce IFNα expression.

75. A method for treating asthma exacerbated by viral infection, comprising administering to a subject in need thereof an ORN of any one of claims 1-54 in an effective amount to treat the asthma exacerbated by viral infection.

76. The method of claim 75 wherein the viral infection is RSV.

77. A method for treating infectious disease, comprising administering to a subject in need thereof an ORN of any one of claims 1-54 in an effective amount to treat the infectious disease.

78. The method of claim 77 wherein the subject has a viral infection.

79. The method of claim 78, wherein the viral infection is hepatitis B.

80. The method of claim 78, wherein the viral infection is hepatitis C.

81. The method of claim 78, further comprising administering an anti-viral agent to the subject.

82. The method of claim 68, wherein the anti-viral agent is linked to the ORN.

83. The method of claim 77, wherein the ORN is delivered by a route selected from the group consisting of oral, nasal, sublingual, intravenous, subcutaneous, mucosal, respiratory, direct injection, and dermally.

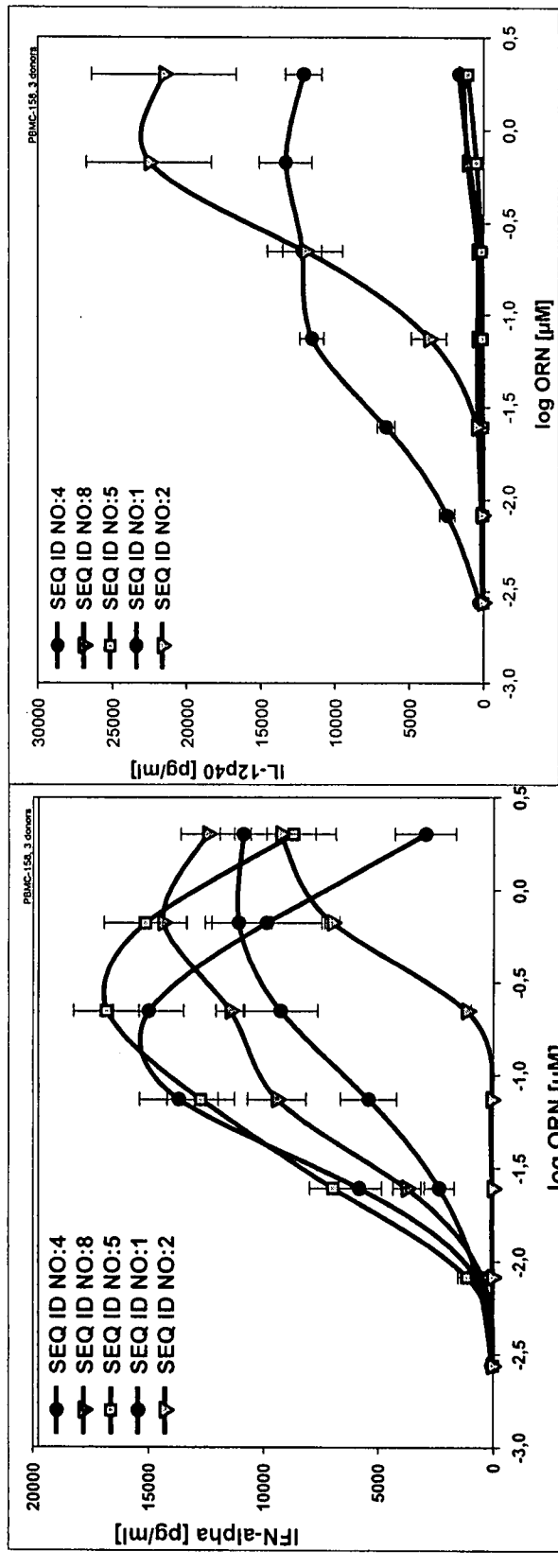


Figure 1A

Figure 1B

Figure 1

Figure 2A

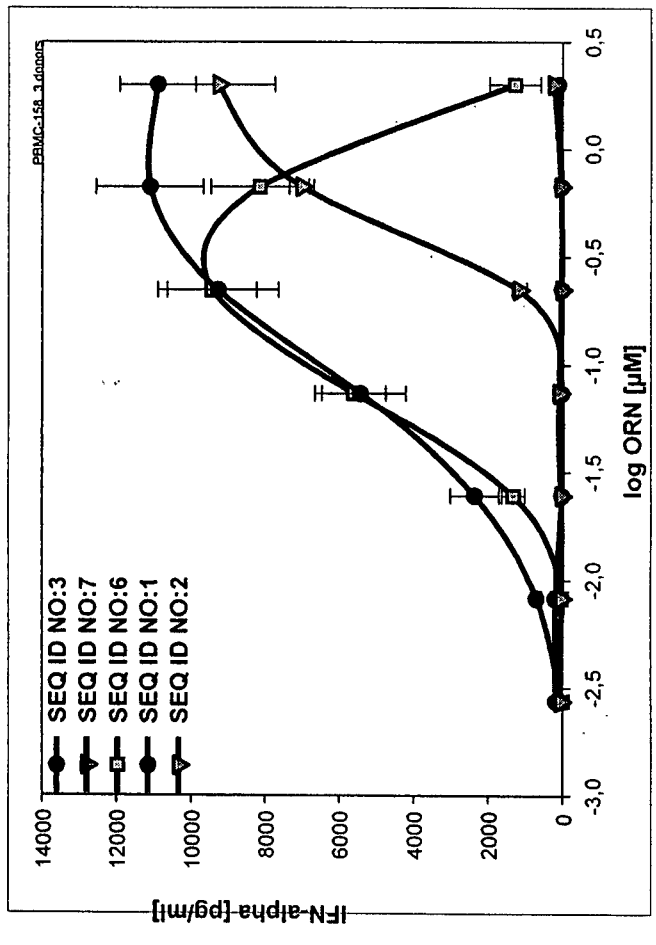


Figure 2B

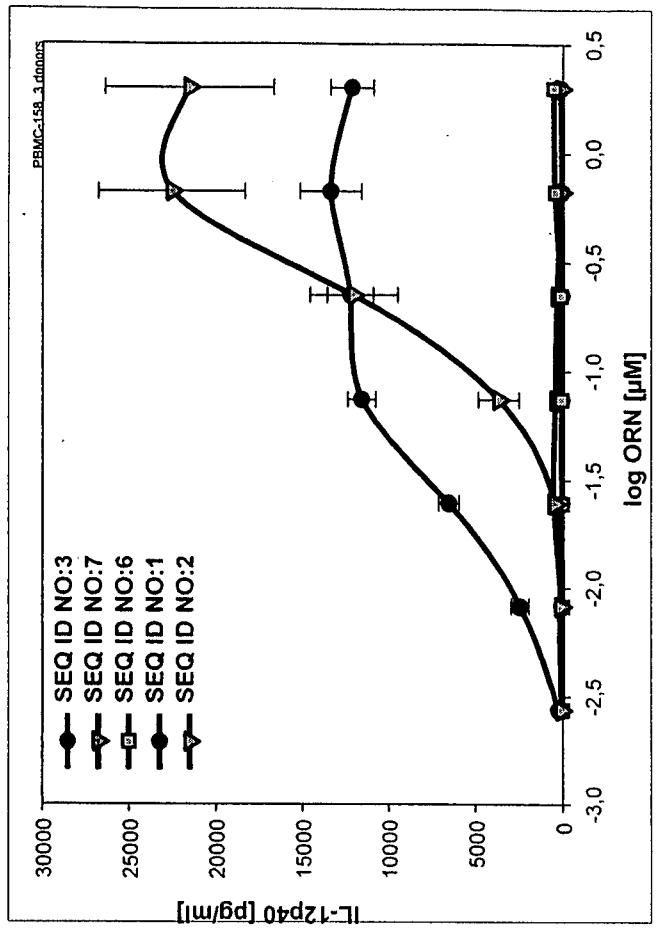


Figure 2

Figure 3B

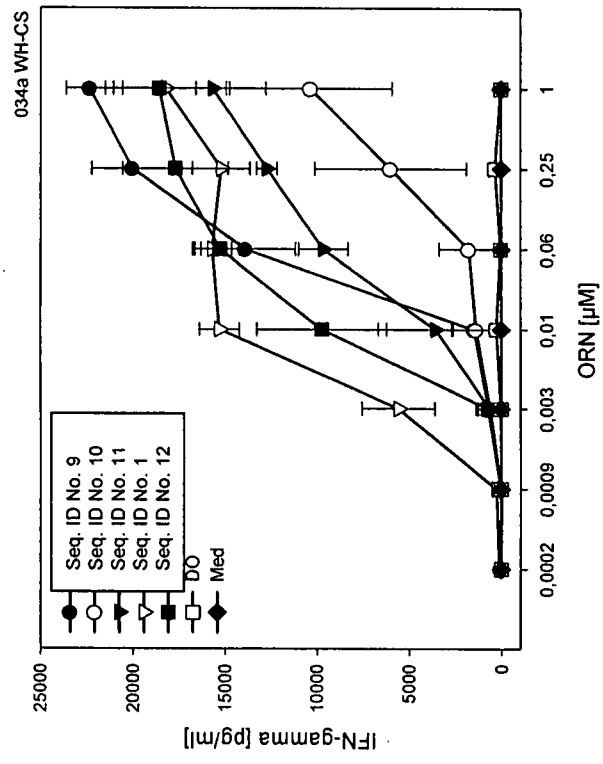


Figure 3A

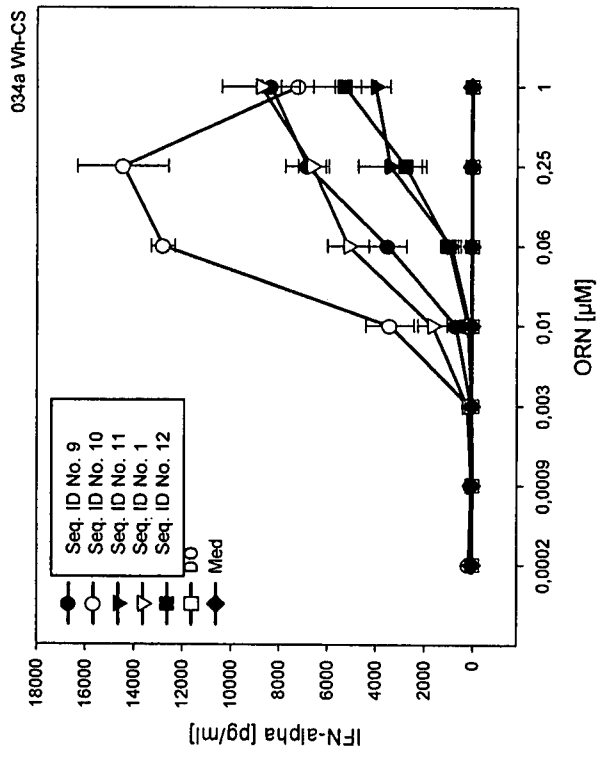


Figure 3

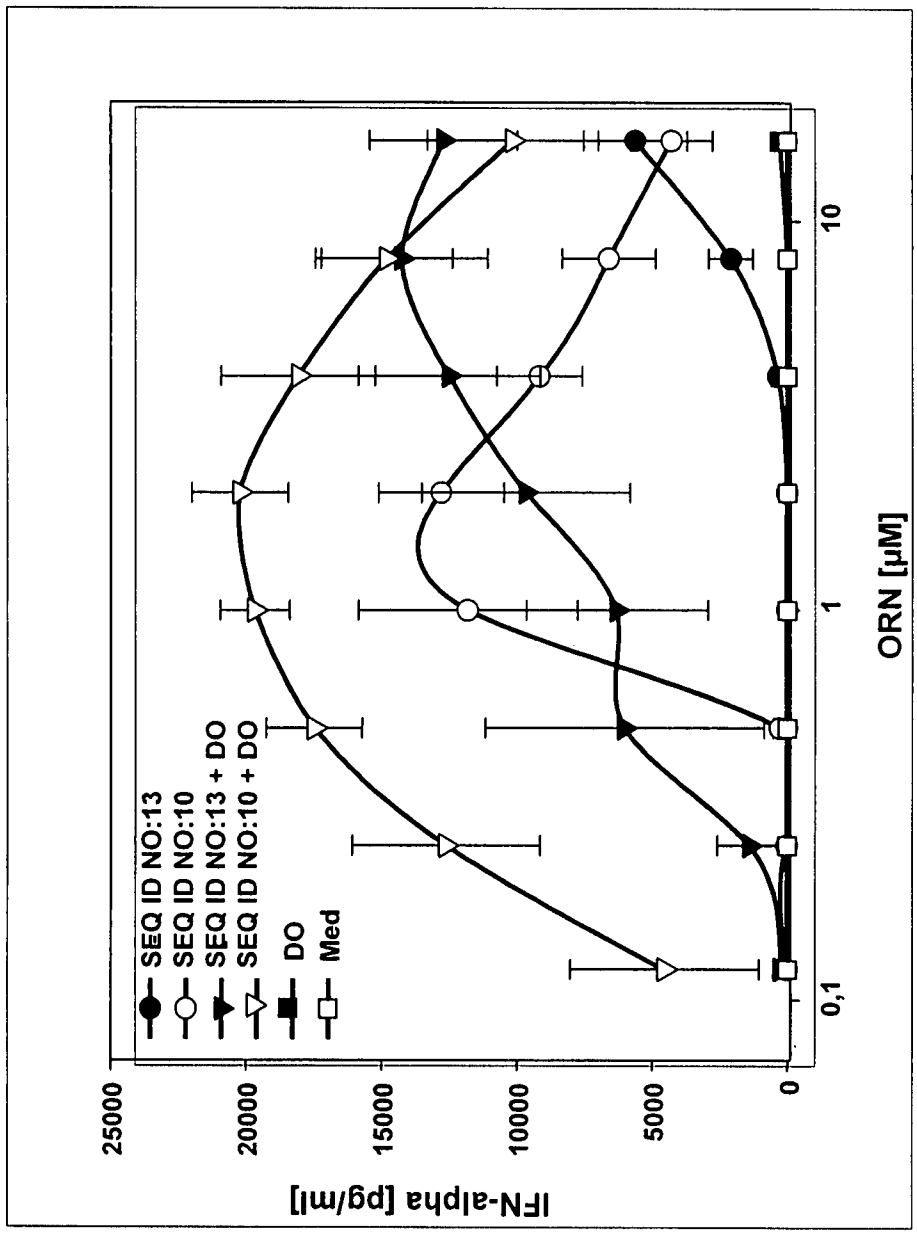

IFN-alpha [pg/ml]
25000
20000
15000
10000
5000
0
0,1
1
10
ORN [µM]
SEQ ID NO:13
SEQ ID NO:10
SEQ ID NO:13 + DO
SEQ ID NO:10 + DO
DO
Med

Figure 4

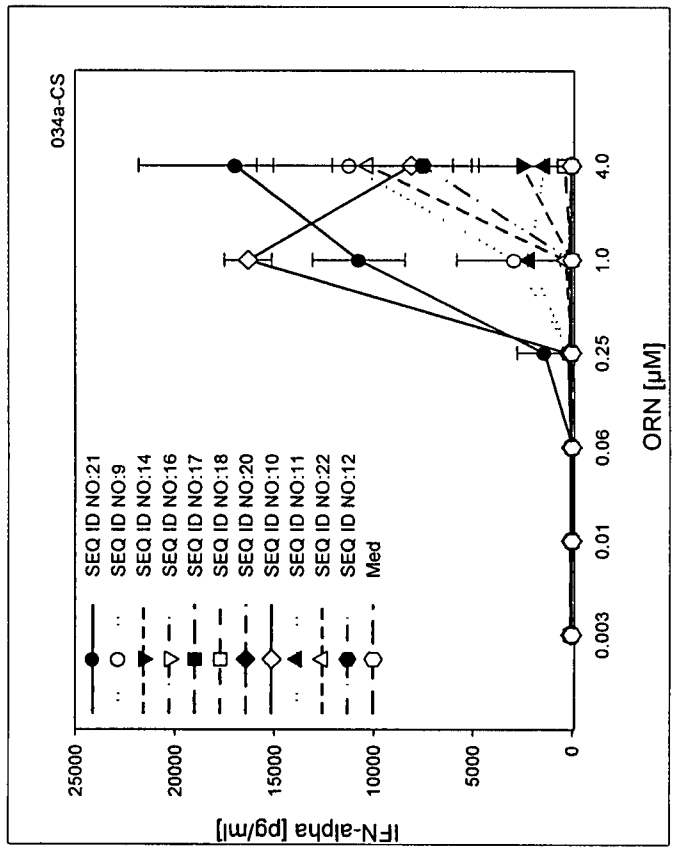

034a-CS
IFN-alpha [pg/ml]
25000
20000
15000
10000
5000
0
ORN [µM]
0.003
0.01
0.06
0.25
1.0
4.0
SEQ ID NO:21
SEQ ID NO:9
SEQ ID NO:14
SEQ ID NO:16
SEQ ID NO:17
SEQ ID NO:18
SEQ ID NO:20
SEQ ID NO:10
SEQ ID NO:11
SEQ ID NO:22
SEQ ID NO:12
Med

Figure 5

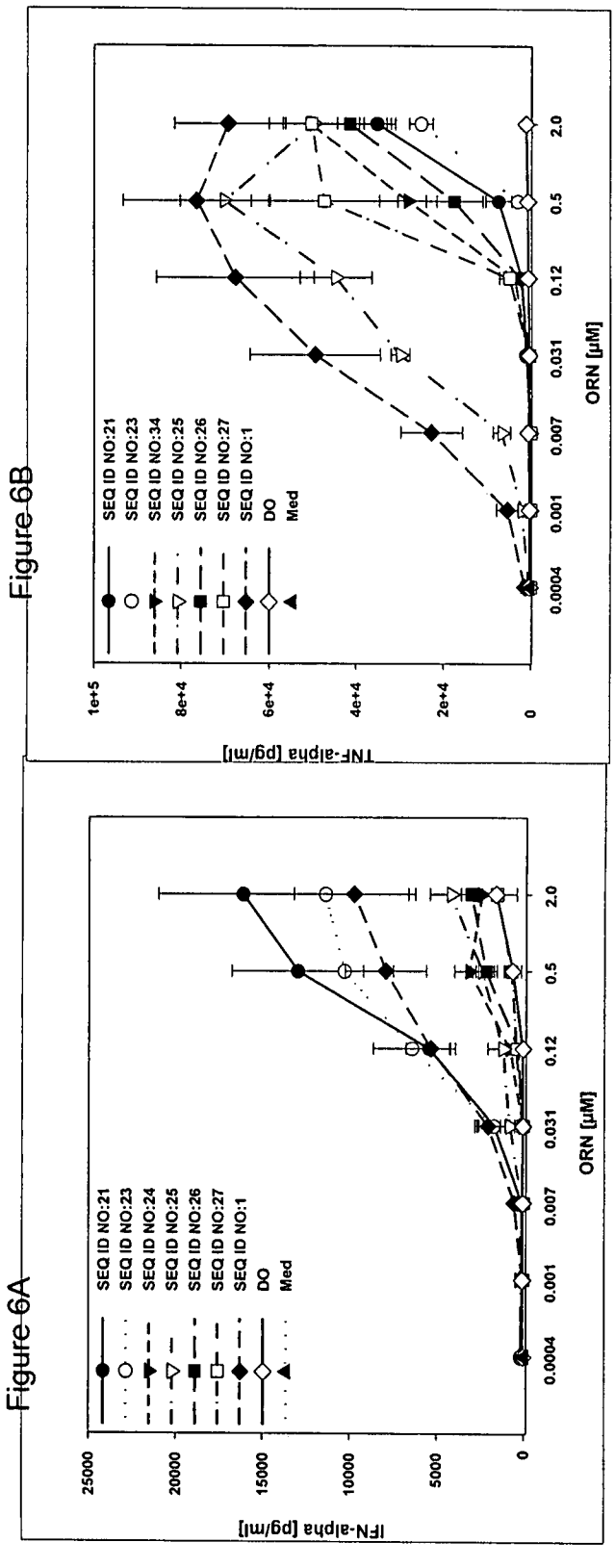

Figure 6A
IFN-alpha [pg/ml]
25000
20000
15000
10000
5000
0
0.0004
0.001
0.007
0.031
0.12
0.5
2.0
ORN [µM]
SEQ ID NO:21
SEQ ID NO:23
SEQ ID NO:24
SEQ ID NO:25
SEQ ID NO:26
SEQ ID NO:27
SEQ ID NO:1
DO
Med
Figure 6B
TNF-alpha [pg/ml]
1e+5
8e+4
6e+4
4e+4
2e+4
0
0.0004
0.001
0.007
0.031
0.12
0.5
2.0
ORN [µM]
SEQ ID NO:21
SEQ ID NO:23
SEQ ID NO:34
SEQ ID NO:25
SEQ ID NO:26
SEQ ID NO:27
SEQ ID NO:1
DO
Med

Figure 6

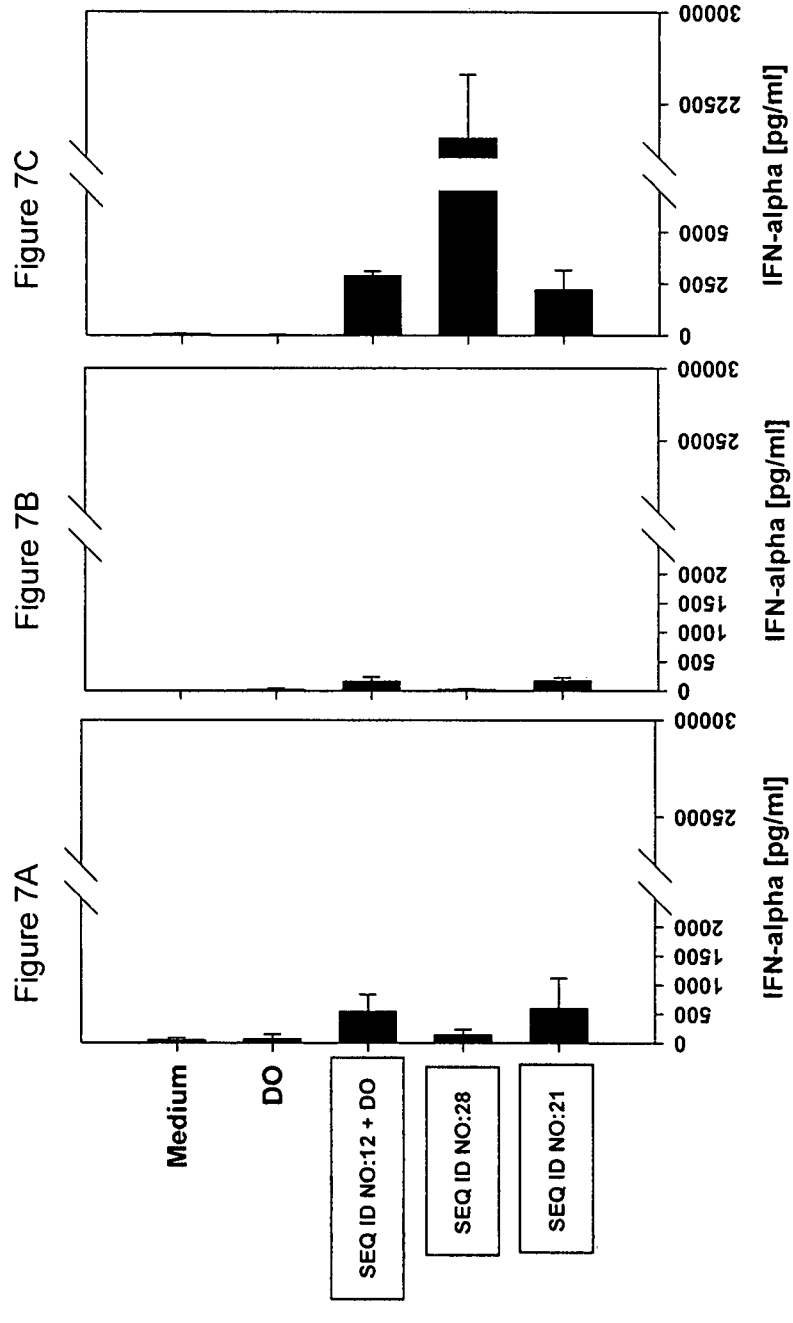

Figure 7C
Figure 7B
Figure 7A
IFN-alpha [pg/ml]
0
500
1000
1500
2000
2500
5000
22500
30000
Medium
DO
SEQ ID NO:12 + DO
SEQ ID NO:28
SEQ ID NO:21

Figure 7

Figure 8A

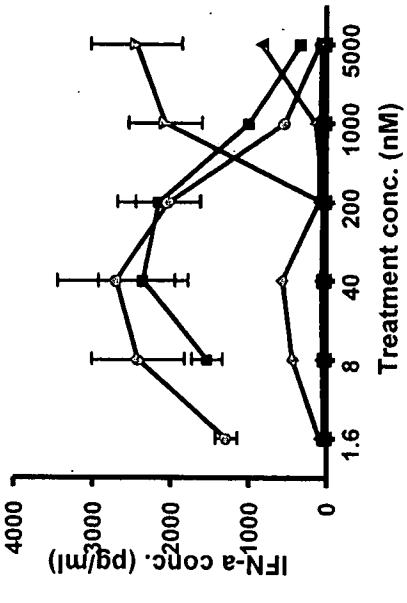


Figure 8B

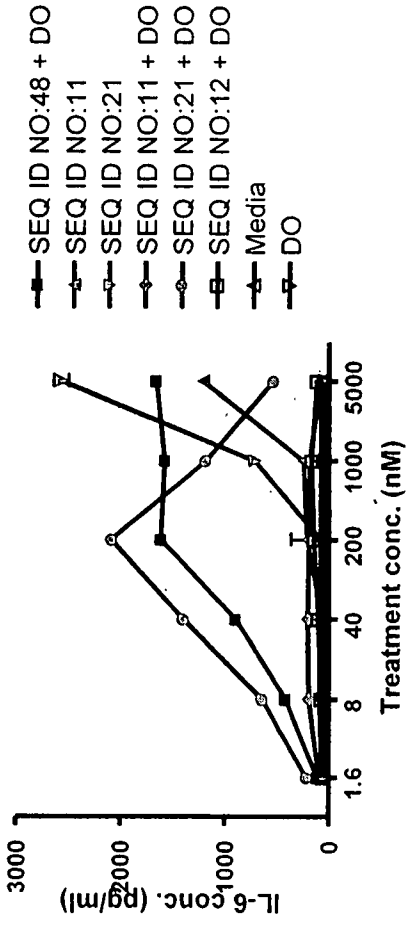


Figure 8C

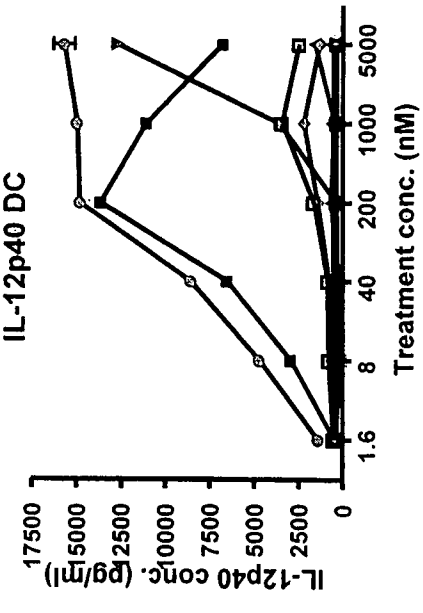


Figure 8D

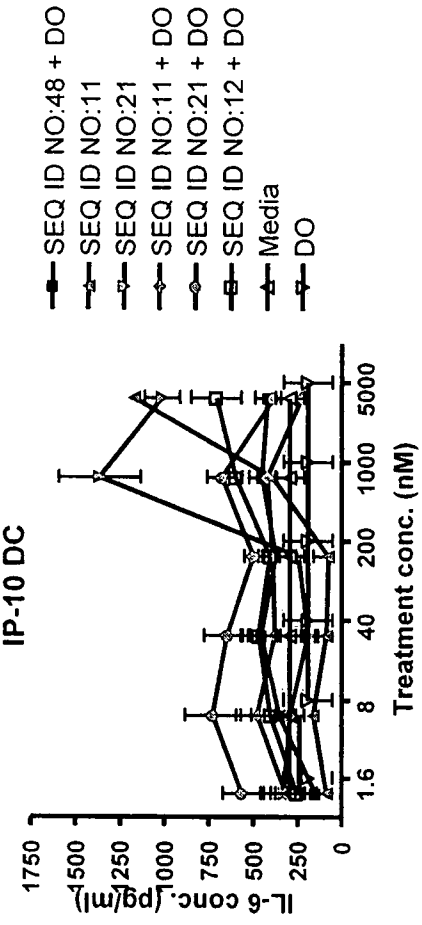


Figure 8

Figure 9A

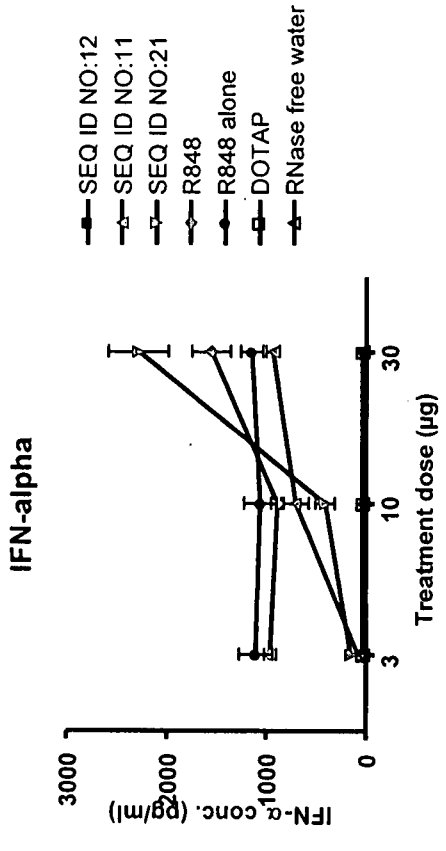


Figure 9B

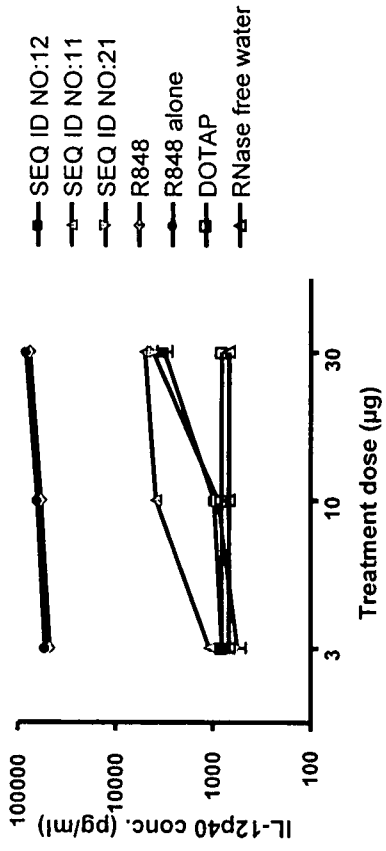


Figure 9A

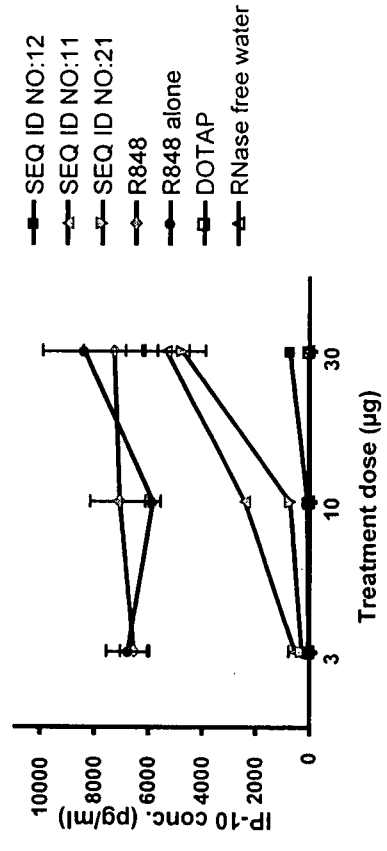


Figure 9D

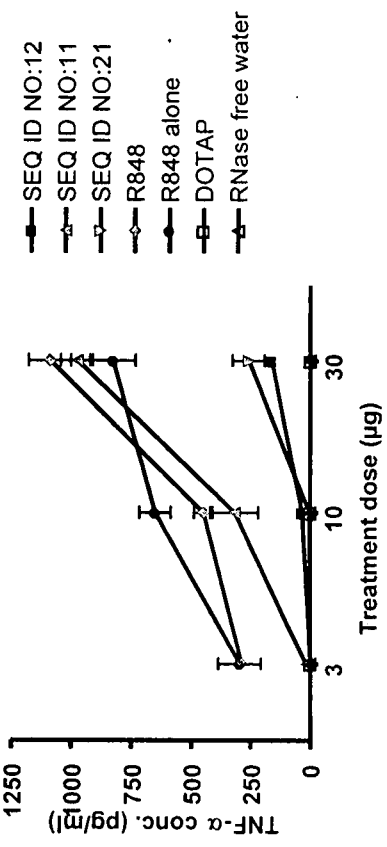


Figure 9

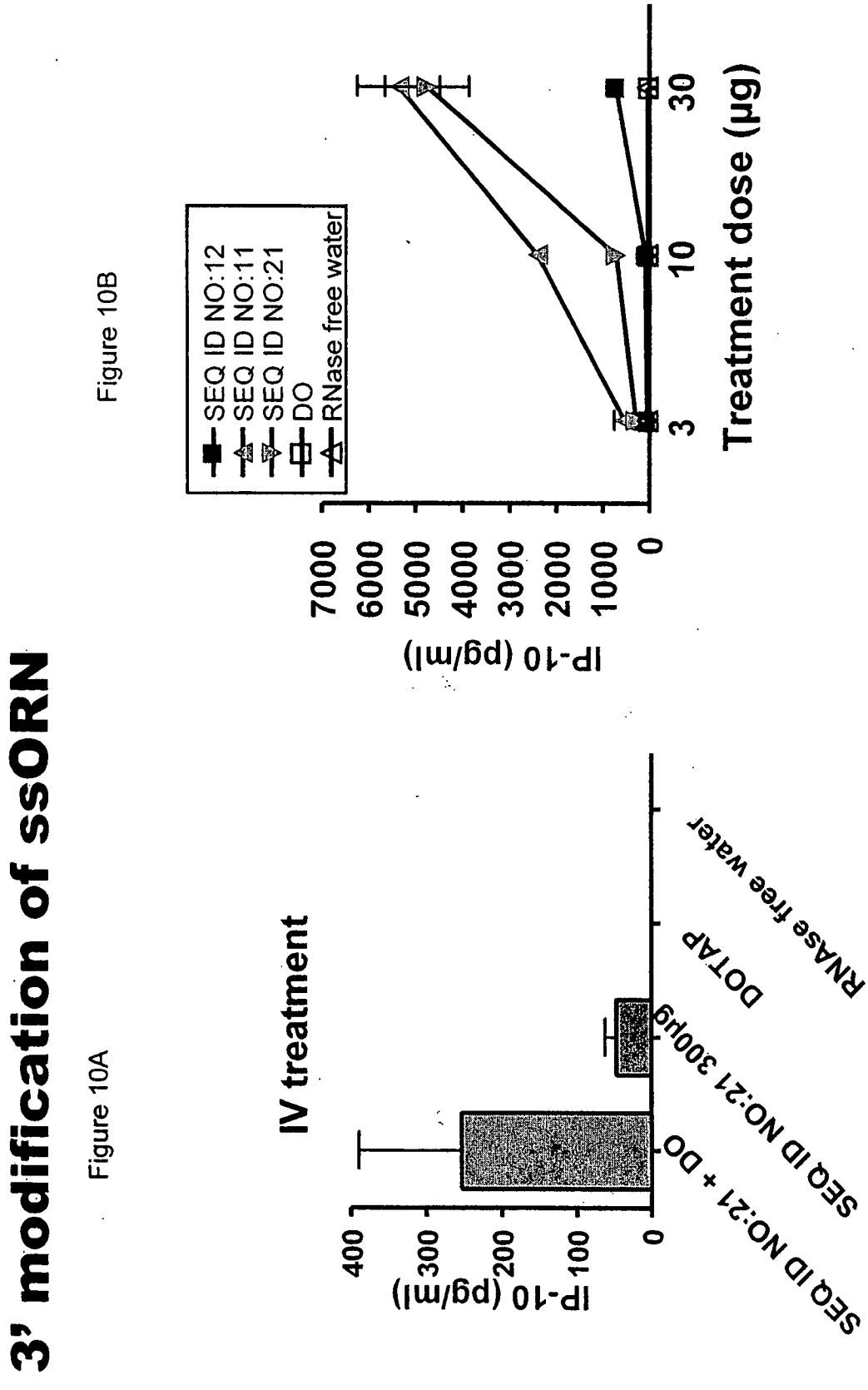

3' modification of ssORN
Figure 10A
IV treatment
IP-10 (pg/ml)
0
100
200
300
400
SEQ ID NO:21 + DO
SEQ ID NO:21 300µg
DOTAP
RNAse free water
Figure 10B
IP-10 (pg/ml)
0
1000
2000
3000
4000
5000
6000
7000
Treatment dose (µg)
3
10
30
SEQ ID NO:12
SEQ ID NO:11
SEQ ID NO:21
DO
RNase free water

Figure 10

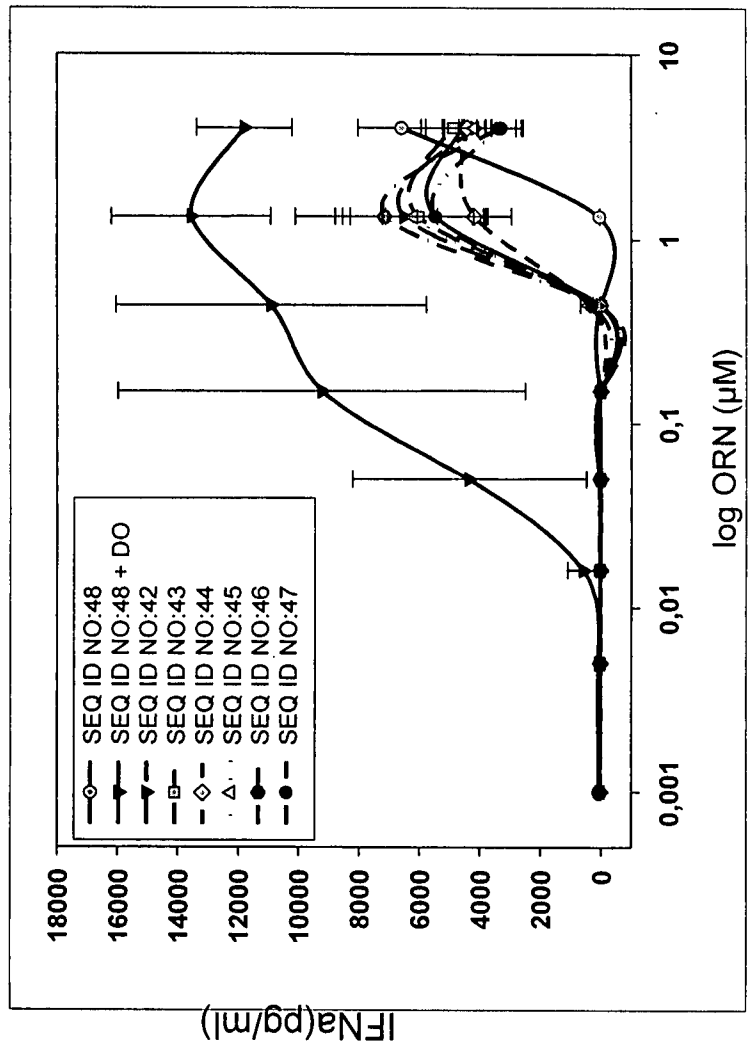

IFNa(pg/ml)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0,001
0,01
0,1
1
10
log ORN (µM)
SEQ ID NO:48
SEQ ID NO:48 + DO
SEQ ID NO:42
SEQ ID NO:43
SEQ ID NO:44
SEQ ID NO:45
SEQ ID NO:46
SEQ ID NO:47

Figure 11