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(54) Title: IMMUNOSTIMULATORY COMPOSITIONS

(57) Abstract: The present disclosure relates to immunostimulatory compositions that are effective in eliciting immune responses in avian species. More specifically, these immunostimulatory compositions comprise an immunomodulator composition and an immunostimulatory oligonucleotide that when administered stimulate toll-like receptor 21.

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IMMUNOSTIMULATORY COMPOSITIONS

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of European Patent Application Nos. EP17207740.6, EP17207746.3, and EP17207750.5, each filed December 15, 2017, the disclosures of which are incorporated herein by reference in their entireties.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created November 30, 2018, is named BHC_168027_SL.txt and is 92,167 bytes in size.

FIELD OF THE INVENTION

[0003] Compositions and methods for stimulating toll-like receptor protein 21 (TLR21) are provided. More specifically, immunostimulatory oligonucleotides and compositions, methods of making immunostimulatory oligonucleotides and compositions, and methods of stimulating TLR21 are disclosed herein.

BACKGROUND OF THE INVENTION

[0004] The immune systems of vertebrates have evolved molecular mechanisms for recognizing invading pathogens and initiating cellular signaling pathways to actively resist infection. Some of the molecular mechanisms are specific for a particular microbe and involve biomolecules such as antibodies that recognize the surface antigens of a single species of pathogen. Unfortunately, pathogen-specific defense mechanisms are not completely effective as some animals do not develop any acquired resistance until after infection has set in, and in some instances, the pathogen has evolved stealthy means for evading a vertebrate's acquired defenses.

[0005] Vertebrates also recognize infections more generally, and this recognition leads to non-specific immune responses such as an uptick in cytokine expression. This defense can be elicited when cellular receptors bind to pathogen-associated molecular patterns (PAMPs). This interaction between the PAMP and the host's cognate receptor for the PAMP can initiate an immune response. For example, toll-like receptor protein 21 (TLR21) is the chicken functional homolog of mammalian TLR9 and is capable of recognizing unmethylated CpG motifs, which have a higher

CpG content in microbes than in vertebrates. Known methodologies leverage this nonspecific immune response pathway by administering plasmids or oligonucleotides having unmethylated CpG motifs, and activation of TLR21 by CpG motif-containing nucleic acids has been shown to activate cellular signals involved in the immune responses to microbial infections. However, administered immunostimulatory plasmids or oligonucleotides alone may fail to elicit a response sufficient to combat infection.

[0006] Large-scale animal producers are in dire need of alternatives to antibiotic treatment of infections. Consumers are pressing these producers for antibiotic-free animal products, and at the same time, increasing incidence of infection due to antibiotic resistant pathogens is illuminating the dangers of administering antibiotics prophylactically to large populations. Similarly, antibiotic resistance is becoming a national emergency in human healthcare. Hospitals and doctors' office are becoming ground zero for the emergence of drug resistance bacteria such as multiple resistance *Staphylococcus aureus* (MRSA).

[0007] Thus, there is a need for immunostimulatory compositions and methods for eliciting non-specific immune responses against pathogens. The disclosed methods and compositions are directed to these and other important needs.

SUMMARY OF THE INVENTION

[0008] Disclosed herein are immunostimulatory compositions comprising an immunomodulator composition comprising a nucleic acid plasmid and a liposomal delivery vehicle; and an immunostimulatory oligonucleotide having at least one CpG motif and a guanine nucleotide-enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide

[0009] Also disclosed herein are methods for preparing an immunostimulatory composition comprising combining an immunomodulator composition comprising a nucleic acid plasmid and an immunostimulatory oligonucleotide to form an immunostimulatory composition, centrifuging the immunostimulatory composition to generate a supernatant and a pellet; and isolating the pellet.

[0010] Further provided are methods of stimulating TLR21 comprising administering an immunostimulatory oligonucleotide and an immunomodulator composition to a subject, wherein the immunostimulatory oligonucleotide comprises at least one CpG motif and a guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide, and wherein

the immunomodulator composition comprises a noncoding nucleic acid plasmid and a cationic lipid delivery vehicle.

[0011] Methods are also disclosed for eliciting an immune response in a subject by administering an immunostimulatory oligonucleotide and an immunomodulator composition, or an immunostimulatory composition comprising an immunostimulatory oligonucleotide and an immunomodulator composition, to a subject, wherein the immunostimulatory oligonucleotide has at least one CpG motif and a guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide, and wherein the immunomodulator composition comprises a noncoding nucleic acid plasmid and a cationic lipid delivery vehicle

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The summary, as well as the following detailed description, is further understood when read in conjunction with the appended drawings. For the purpose of illustrating the disclosed compositions and methods, there are shown in the drawings exemplary embodiments of the compositions and methods; however, the compositions and methods are not limited to the specific embodiments disclosed. In the drawings:

[0013] **FIG. 1** illustrates the chemical structure of a cholesteryl moiety attached to a tetraethylene glycol linker.

[0014] **FIGs. 2A and 2B** compare the immunogenicities of immunostimulatory plasmid DNA, plasmid DNA complexed with cationic liposomes, and immunostimulatory oligonucleotides. **FIG. 2A** compares the immunogenicities of an immunostimulatory plasmid DNA ("pDNA") and pDNA complexed with cationic liposomes ("pDNA-F"). **FIG. 2B** compares the immunogenicities of pDNA, pDNA-F, and immunostimulatory oligonucleotide GCGT3-TG4T having a 5'-cholesteryl modification ("5Chol-GCGT3-TG4T").

[0015] **FIGs. 3A and 3B** compare the immunogenicities of immunostimulatory plasmid DNA, immunostimulatory plasmid DNA complexed with cationic liposomes, immunostimulatory oligonucleotides, and combinations thereof. **FIG. 3A** compares the immunogenicities of pDNA, pDNA-F, 5Chol-GCGT3-TG4T, pDNA combined with 5'Chol-GCGT3-TG4T ("pDNA-5Chol-GCGT3-TG4T"), and pDNA-F combined with 5Chol-GCGT3-TG4T ("pDNA-F-5Chol-GCGT3-TG4T"), wherein the immunostimulatory oligonucleotides are at nM concentrations and pDNA and pDNA-F are at μg/ml concentrations. **FIG. 3B** depicts the differences in immunogenicity between pDNA, pDNA-F, 5Chol-GCGT3-TG4T, pDNA combined with 5'Chol-GCGT3-TG4T ("pDNA-

5Chol-GCGT3-TG4T"), and pDNA-F combined with 5Chol-GCGT3-TG4T ("pDNA-F-5Chol-GCGT3-TG4T"), wherein the immunostimulatory oligonucleotides are at pM concentrations and pDNA and pDNA-F are at ng/ml concentrations;

[0016] **FIG. 4** illustrates the ability of pDNA-F fractions to stimulate TLR21-mediated immune responses in HEK293-bsd-cTLR21 cells. Specifically, the immunogenicity of pDNA-F stored at 4°C was compared to that of pDNA-F obtained in the pellet ("pDNA-F pellet") and the supernatant ("pDNA-F supernatant") of a centrifuged sample.

[0017] **FIGs. 5A and 5B** graphically depict the ability to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells of pDNA-F-5Chol-GCGT3-TG4T and 5Chol-GCGT3-TG4T at high and low concentrations, respectively.

[0018] **FIGs. 6A and 6B** compare the ability of high and low concentrations, respectively, of pDNA-F-5Chol-GCGT3-TG4T to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of 5Chol-GCGT3-TG4T obtained in the pellet ("pDNA-F 5Chol pellet") and supernatant ("pDNA-F 5Chol Uberstand") of a centrifuged pDNA-F sample.

[0019] **FIGs. 7A and 7B** compare the ability of high and low concentrations, respectively, of 5Chol-GCGT3-TG4T to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of 5Chol-GCGT3-TG4T obtained in the pellet ("5Chol pellet") and supernatant ("5Chol Uberstand") of a centrifuged pDNA-F sample.

[0020] **FIGs. 8A and 8B** compare the ability of high and low concentrations, respectively, of 5Chol-GCGT3-TG4T ("5Chol-GCGT3-TG4T 4°C") to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of pDNA-F combined with 5Chol-GCGT3-TG4T "pDNA-F/5-Chol-GCGT3-TG4T").

[0021] **FIGs. 9A and 9B** compare the ability of high and low concentrations, respectively, of pDNA-F combined with 5Chol-GCGT3-TG4T ("pDNA-F/5-Chol-GCGT3-TG4T") to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of pDNA-F combined with 5Chol-GCGT3-TG4T obtained in the pellet ("pDNA-F/5-Chol-GCGT3-TG4T pellet") and supernatant ("pDNA-F/5-Chol-GCGT3-TG4T supernatant") of a centrifuged pDNA-F sample.

[0022] **FIG.s 10A and 10B** compare the ability of high and low concentrations, respectively, of pDNA-F combined with 5Chol-GCGT3-TG4T ("pDNA-F-5-Chol-GCGT3-TG4T") to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of pDNA-F and immunostimulatory oligonucleotide 5-Chol-GCGT3-TG4T.

[0023] **FIGs. 11A and 11B** compare the ability of high and low concentrations, respectively, of pDNA-F combined with 5Chol-GCGT3-TG4T ("pDNA-F-5-Chol-GCGT3-TG4T") to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of pDNA-F combined with 5Chol-GCGT3-TG4T obtained in the pellet ("pDNA-F-5-Chol-GCGT3-TG4T pellet") and supernatant ("pDNA-F-5-Chol-GCGT3-TG4T") of a centrifuged pDNA-F sample.

[0024] **FIGs. 12A and 12B** compare the ability of high and low concentrations, respectively, of pDNA-F, immunostimulatory oligonucleotide GCGT3-TG4T, and pDNA-F complexed with GCGT3-TG4T ("pDNA-F-GCGT3-TG4T") to generate TLR21-mediated immune responses in HEK293-bsd-cTLR21 cells.

[0025] **FIGs. 13A and 13B** compare the ability of high and low concentrations, respectively, of pDNA-F combined with immunostimulatory oligonucleotide GCGT3-TG4T ("pDNA-F-GCGT3-TG4T") to generate a TLR21-mediated immune response in HEK293-bsd-cTLR21 cells to that of pDNA-F combined with immunostimulatory oligonucleotide GCGT3-TG4T obtained in the pellet ("pDNA-F-GCGT3-TG4T pellet") and supernatant ("pDNA-F-GCGT3-TG4T supernatant") of a centrifuged pDNA-F sample.

[0026] **FIG. 14** depicts mean Haemagglutination inhibition (HI) titres (Log2) (with standard deviation) results for ODN1 (GCGT3-TG4T-5Chol) at days 14 (top panel) and 21 (bottom panel) post vaccination (pv). Asterisks indicate the level of significance (*=significant to ****=highly significant).

[0027] **FIG. 15** depicts mean HI titres (Log2) (with standard deviation) results for ODN1 (GCGT3-TG4T-5Chol) during the entire study.

[0028] **FIG. 16** depicts mean HI titres (Log2) (with standard deviation) results for ODN2 (GCGT3-TG4T) at days 14 (top panel) and 21 (bottom panel) post vaccination. Asterisks indicate the level of significance (*=significant to ****=highly significant).

[0029] **FIG. 17** depicts mean HI titres (Log2) (with standard deviation) results for ODN2 (GCGT3-TG4T) during the entire study.

[0030] **FIG. 18** depicts mean HI titres (Log2) (with standard deviation) results for ODN3 (2006-PTO) at days 14 (top panel) and 21 (bottom panel) post vaccination. Asterisks indicate the level of significance (*=significant to ****=highly significant).

[0031] **FIG. 19** depicts mean HI titres (Log2) (with standard deviation) results for ODN3 (2006-PTO) during the entire study.

[0032] **FIG. 20** depicts mean HI titres (Log2) (with standard deviation) results for positive and negative control Test Articles at days 14 (top panel) and 21 (bottom panel) post vaccination. Asterisks indicate the level of significance (*=significant to ****=highly significant).

[0033] **FIG. 21** depicts mean HI titres (Log2) (with standard deviation) results for positive and negative control Test Articles during the entire study.

[0034] **FIG. 22** depicts mean HI titres (Log2) (with standard deviation) results at the most optimal concentrations of ODNs during the entire study compared to NDV vaccine alone.

[0035] **FIG. 23** depicts mean HI titres (Log2) (with standard deviation) results at the most optimal concentrations of ODNs at day 14 (top panel) and 21 (bottom panel) pv compared to NDV vaccine alone.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0036] The disclosed compositions and methods may be understood more readily by reference to the following detailed description taken in connection with the accompanying figures, which form a part of this disclosure. It is to be understood that the disclosed compositions and methods are not limited to the specific compositions and methods described and/or shown herein, and that the terminology used herein is for the purpose of describing particular embodiments by way of example only and is not intended to be limiting of the claimed compositions and methods.

[0037] Unless specifically stated otherwise, any description as to a possible mechanism or mode of action or reason for improvement is meant to be illustrative only, and the disclosed compositions and methods are not to be constrained by the correctness or incorrectness of any such suggested mechanism or mode of action or reason for improvement.

[0038] Throughout this text, the descriptions refer to compositions and methods of using said compositions. Where the disclosure describes or claims a feature or embodiment associated with a composition, such a feature or embodiment is equally applicable to the methods of using said composition. Likewise, where the disclosure describes or claims a feature or embodiment associated with a method of using a composition, such a feature or embodiment is equally applicable to the composition.

[0039] When a range of values is expressed, another embodiment includes from the one particular value and/or to the other particular value. Further, reference to values stated in ranges include each and every value within that range. All ranges are inclusive and combinable. When values are expressed as approximations, by use of the antecedent “about,” it will be understood that

the particular value forms another embodiment. Reference to a particular numerical value includes at least that particular value, unless the context clearly dictates otherwise.

[0040] It is to be appreciated that certain features of the disclosed compositions and methods which are, for clarity, described herein in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the disclosed compositions and methods that are, for brevity, described in the context of a single embodiment, may also be provided separately or in any subcombination.

[0041] As used herein, the singular forms "a," "an," and "the" include the plural.

[0042] "Co-administered" as used herein refers to administering the immunomodulatory composition in combination with the immunostimulatory oligonucleotide to achieve the desired immunostimulatory effect. The immunomodulatory composition and the immunostimulatory oligonucleotide can be co-administered as separate compositions or together as a single composition. If the immunomodulatory composition and the immunostimulatory oligonucleotide are separate compositions, they can be co-administered either simultaneously or sequentially in either order. For sequential co-administration, there may be a delay of a minute, an hour, or even one or more days between the administration of the immunomodulatory composition and the immunostimulatory oligonucleotide.

[0043] As used herein, "fusing" refers to creating a chemical bond between two chemically reactive species. In the context of this disclosure, fusing most often refers to incorporating specific elements into an oligonucleotide. For example, a run of thymine nucleotides can be fused to the 3' end of an oligonucleotide.

[0044] As used herein, "G-quartet sequence" refers to a stretch of consecutive guanine residues near the 5' end of an oligonucleotide that enables the oligonucleotide to interact with other G-quartet sequences to form a G-quartet. The G-quartet enhances the immunostimulatory properties of the nucleic acid. For example, oligonucleotides comprising G-quartet sequences may interact, resulting in G-quartets. G-quartet sequences occurring in the promoter region of a gene may form quaternary structures involved in regulating the expression of the gene. While a G-quartet sequence is not limited to any particular sequence, an example of a G-quartet sequence is TGGGGT.

[0045] As used herein, "G-wire sequence," "G wire sequence," "Gwire sequence," and related terms, refer to a plurality, most often two, of at least four consecutive guanine nucleotides. The pluralities of guanine nucleotides, located at or near the 5' terminus of an oligonucleotide, are

separated by two or more non-guanine nucleotides (i.e., thymine). G-wire sequences are capable of interacting with other G-wire sequences to form a G-wire structure. A G-wire structure can enhance the immunostimulatory properties of a nucleic acid. An exemplary G-wire sequence is GGGGTTGGGG (SEQ ID NO: 257) or GGGGTTGGGGTTTT (SEQ ID NO: 258).

[0046] As used herein, the terms "guanine nucleotide enriched sequence," "guanine enriched sequence," and the like, refer to sequences comprising either a run of consecutive guanine nucleotides, usually between four to six guanine nucleotides, or a region of a nucleic acid, typically at or near the 5' end of an oligonucleotide having more guanine nucleotides than adenine, cytosine, or thymine nucleotides. A guanine enriched sequence as disclosed herein can enhance the immunostimulatory properties of an oligonucleotide. G-quartet and G-wire sequences are both types of guanine nucleotide enriched sequences.

[0047] The term "immunomodulatory composition" as used herein refers to a composition comprising at least an immunogenic nucleic acid plasmid and a liposomal delivery vehicle. In some aspects of the presently disclosed compositions and methods, the nucleic acid plasmid may not code for a particular immunogen and may be immunogenic based on the inherent properties of the nucleic acid plasmid. In some aspects, the liposomal delivery vehicle is cationic.

[0048] An "immunogenic nucleic acid plasmid" is a nucleic acid plasmid that, when detected by a vertebrate immune system, elicits an immune response. Some immunogenic nucleic acid plasmids comprise an increased percentage of CpG dinucleotide motifs compared to nucleic acid plasmid sequences naturally occurring in some vertebrate organisms. Without being bound to theory, it is believed that increased CpG dinucleotide motifs are present in bacterially derived nucleic acid, and therefore, such CpG-enriched nucleic acid appears foreign to host immune defenses. Immunogenic nucleic acid plasmids can comprise non-naturally occurring nucleotides and derivatives of nucleotides.

[0049] "Immunostimulatory composition" as used herein refers to a composition comprising an immunomodulatory composition and an immunostimulatory oligonucleotide. In some aspects, the immunostimulatory oligonucleotide and the immunomodulatory composition comprise a single formulation that is the immunostimulatory composition. In some aspects, the immunostimulatory oligonucleotide may be physically associated with the liposomal delivery vehicle of the immunomodulatory composition.

[0050] As used herein, "inserting" refers to adding specific nucleotide(s) at specific positions during the synthesis of an oligonucleotide.

[0051] As used herein, "parallel orientation" refers to the directional interaction between different oligonucleotides. For example, individual oligonucleotides oriented in the same 5' to 3' direction are in a parallel orientation.

[0052] As used herein, "percent identity" and like terms are used to describe the sequence relationships between two or more nucleic acids, polynucleotides, proteins, or polypeptides, and are understood in the context of and in conjunction with the terms including: (a) reference sequence, (b) comparison window, (c) sequence identity and (d) percentage of sequence identity.

(a) A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) A "comparison window" includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions, substitutions, or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions, substitutions, or deletions) for optimal alignment of the two sequences. Those of skill in the art understand that to avoid a misleadingly high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

(c) Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, Adv. Appl. Math., 2: 482, 1981; by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol., 48: 443, 1970; by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 8: 2444, 1988; by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, Calif., GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 7 Science Dr., Madison, Wis., USA; the

CLUSTAL program is well described by Higgins and Sharp, Gene, 73: 237-244, 1988; Corpet, et al., Nucleic Acids Research, 16:881-90, 1988; Huang, et al., Computer Applications in the Biosciences, 8:1-6, 1992; and Pearson, et al., Methods in Molecular Biology, 24:7-331, 1994. The BLAST family of programs which may be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, Current Protocols in Molecular Biology, Chapter 19, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York, 1995. New versions of the above programs or new programs altogether will undoubtedly become available in the future, and may be used with the present disclosure.

(d) "Percent identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions, substitutions, or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions, substitutions, or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0053] "Therapeutically effective amount" refers to an amount of an immunomodulatory composition and/or an immunostimulatory oligonucleotide or of an immunostimulatory composition that treats the subject.

[0054] "Synergistically effective amount" refers to an amount of an immunomodulatory composition and an immunostimulatory oligonucleotide that provides a synergistic, or more than additive, effect in treating the subject. The term "subject" as used herein is intended to mean any animal, but in particular, avian species. "Avian species" includes, but is not limited to, chickens, domestic turkeys, waterfowl and any other food source fowl.

[0055] As used herein, "treating" and like terms refer to reducing the severity and/or frequency of symptoms of an infection, eliminating symptoms and/or the underlying cause of said symptoms, reducing the frequency or likelihood of symptoms and/or their underlying cause, and/or improving or remediating damage caused, directly or indirectly, by an infectious agent.

[0056] Various terms relating to aspects of the description are used throughout the specification and claims. Such terms are to be given their ordinary meaning in the art unless otherwise indicated. Other specifically defined terms are to be construed in a manner consistent with the definitions provided herein.

[0057] Immunostimulatory compositions are provided herein comprising an immunomodulator composition comprising a nucleic acid plasmid and a liposomal delivery vehicle and an immunostimulatory oligonucleotide having at least one CpG motif and an guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide.

[0058] Immunostimulatory oligonucleotides, as described herein, can interact with TLR21 to elicit an immune response. The immunostimulatory oligonucleotides comprise at least one unmethylated dinucleotide CpG motif, which interacts with pathogen recognition receptors expressed in the host organism. The immunostimulatory oligonucleotides also have a guanine nucleotide enriched sequence. These sequences can facilitate the folding of a DNA strand into a quaternary structure or promote the aggregation of one or more immunostimulatory oligonucleotides that have an enhanced guanine the sequence. The guanine enriched sequence need not be comprised solely of guanine nucleotides, but it must be enriched. A guanine enriched sequence, as described *supra* and exemplified throughout these disclosures, typically is located at or near (within four nucleotides of) the oligonucleotide terminus. Additional manipulation of the oligonucleotide sequence and structure can further enhance the immunostimulatory oligonucleotide's ability to stimulate TLR21. Therefore, one embodiment of the present disclosure comprises an immunostimulatory composition comprising at least one immunostimulatory oligonucleotide having at least one CpG motif and a guanine enriched sequence beginning at or within four nucleotides of the 5' terminus of the immunostimulatory oligonucleotide.

[0059] In some aspects of the present disclosure, the addition of guanine nucleotide runs to the 5' end of the CpG containing immunostimulatory oligonucleotide can significantly improve immunogenicity of the immunostimulatory oligonucleotide. Not only does the position of the guanine rich sequence in the immunostimulatory oligonucleotide affect enhancement of TLR21

activation, but the content of the sequence has an effect as well. For this reason, in some aspects of the present disclosure, guanine enriched sequences comprise a plurality of consecutive guanine nucleotides.

[0060] In some embodiments, the guanine enriched sequence comprises a first plurality of consecutive guanine nucleotides. In some aspects the first plurality of guanine nucleotides comprises two to eight guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises two guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises three guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises four guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises five guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises six guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises seven guanine nucleotides. In some aspects, the first plurality of guanine nucleotides comprises eight guanine nucleotides. In still other aspects, the first plurality of guanine nucleotides comprises more than eight guanine nucleotides.

[0061] In some embodiments of the present invention, the oligonucleotide comprises SEQ ID NO:16, 17, 18, 19, 20, 21, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 77, 78, 81, 82, 85, 86, 89, 90, 92, 93, 96, 97, 100, 102, 104, 106, 108, or 143. In other embodiments, the guanine enriched sequence comprises TTAGGG, TTAGGGTTAGGG (SEQ ID NO:261), TTTTGGGG, GGGGTTTT, GGGGTTTTGGGG (SEQ ID NO:262), TTAGGG, TTAGGGTTAGGGTTTT (SEQ ID NO:263), TGTGGGTGTGTGTGGG (SEQ ID NO:269), GGAGG, TGGAGGC, or TGGAGGCTGGAGGC (SEQ ID NO:264). In still other embodiments, the oligonucleotide comprises SEQ ID NO:110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 124, 125, 126, 127, 129, 130, 131, 134, 136, 137, or 138.

[0062] A single run of guanine nucleotides is not the only 5' modification that can enhance TLR21 stimulation. For example, adenine, cytosine, and thymine enriched sequences can also be added to the 5' end of an oligonucleotide having CpG motif and result in enhanced TLR21 stimulation, albeit less than that elicited by the guanine enriched sequences at the 5' end of the oligonucleotide. While a single plurality of guanine residues at the 5' end of the oligonucleotide can elicit TLR21 stimulation, additional pluralities of guanine nucleotides in the guanine enriched sequence may further enhance the stimulatory properties of the oligonucleotide. Thus, in some

aspects, the oligonucleotide of the present disclosure comprises a second plurality of guanine nucleotides between the first plurality of guanine nucleotides and the at least one CpG motif.

[0063] In some aspects, a plurality of guanine nucleotides comprises a G-quartet sequence. In some embodiments, the first plurality of guanine nucleotides, the second plurality of guanine nucleotides, or both comprise a G-quartet sequence. G-quartet sequences, as defined above, also allow for interaction between oligonucleotides. Without being bound by theory, interaction at the 5' end of the oligonucleotides allows for the concentration of CpG dinucleotide motifs and a corresponding enhanced opportunity for recognition by TRL21. In some embodiments, the immunostimulatory composition further comprises at least one additional oligonucleotide having a G-quartet sequence, wherein the oligonucleotide and the at least one additional oligonucleotide have a parallel orientation in a quaternary structure. In some aspects, the G-quartet sequence comprises TGGGGT.

[0064] Another guanine enriched sequence that can be added at or near the 5' terminus of an oligonucleotide having CpG motifs is a G-wire sequence. In some aspects of the present disclosure, the first and second pluralities of guanine nucleotides comprise a G-wire sequence. In some aspects, the G-wire sequence comprises SEQ ID NO:257 or 258. In still other aspects, the G-wire sequence comprises SEQ ID NO:141, 142, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, or GCGT-Gwire3. The two pluralities of guanine nucleotides can be separated by non-guanine nucleotides, nucleotide analogs, or any other spacer or linker. For example, in some aspects of the present disclosure, the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least one nucleotide. As used herein, the term "spacer" refers to a chemical linkage between similar nucleotide motifs, i.e., between two CpG motifs or between two guanine nucleotide enriched sequence motifs, whereas the term "linker" refers to a chemical linkage between different nucleotide motifs, i.e., between a guanine nucleotide enriched sequence and another nucleotide motif, e.g., a CpG motif. The terms "spacer" and "linker" are used for clarity in describing which aspect of an oligonucleotide is being discussed. However, it will be understood by those skilled in the art that the structures disclosed herein for spacers can be interchangeable with the structures disclosed herein for linkers, and vice versa.

[0065] Without being bound by any particular theory, it is possible that the G-wire sequence enables an oligonucleotide to interact and aggregate with other oligonucleotides having G-

wire sequences. The conformation assumed by the aggregation of oligonucleotides having G-wire sequences is referred to as G-wire conformation, and this accumulation of oligonucleotides and their CpG motifs can lead to enhanced stimulation of TLR21.

[0066] The guanine enriched sequences may be separated from the CpG nucleotide motifs by nucleotides, nucleotide analogs, or other linkers. Therefore, in some embodiments of the present disclosure, the oligonucleotide further comprises a linker between the guanine enriched sequence and the downstream at least one CpG motif. As used herein, "downstream" means in the 5' → 3' direction; i.e., a "downstream" nucleotide or motif is a nucleotide or motif that is 3' of a comparison sequence element. "Upstream" means in the 3' → 5' direction; i.e., an "upstream" nucleotide or motif is a nucleotide or motif that is 5' of a comparison sequence element. The linker need not be directly adjacent to either the guanine enriched sequence or the CpG motif; rather, the linker must reside between the two sequence motifs regardless of intervening sequences between the guanine enriched sequence and the linker, as well as between the CpG motif and the linker. In some embodiments of the present disclosures, the linker comprises at least three nucleotides. The linker also may not comprise nitrogenous bases. For example, in some aspects, the linker is a hexaethyleneglycol, a propanediol, a triethyleneglycol, or derivatives thereof. In other examples, the oligonucleotide having a linker comprises 2006-PDE5dG4-X1 or 2006-PDE5dG4-X3.

[0067] Dinucleotide CpG motifs present in the oligonucleotides of the present disclosure are believed to be PAMPs recognized by TLR21 in chickens. While even a single CpG motif can stimulate TLR21, multiple CpGs present on an oligonucleotide can increase stimulated TLR21 signal strength. For this reason, in some aspects of the present invention, the at least one CpG motif comprises two, three, four, or five CpG motifs. In some aspects the at least one CpG motif comprises six or more CpG motifs. In some aspects, the at least one CpG motif comprises two CpG motifs. In some aspects, the at least one CpG motif comprises three CpG motifs. In some aspects, the at least one CpG motif comprises four CpG motifs. In some embodiments, the at least one CpG motif comprises four CpG motifs.

[0068] In some embodiments of the presently disclosed oligonucleotides, each CpG motif may be separated from the other CpG motifs by at least one nucleotide or nucleotide analog. In some aspects, the at least one nucleotide is two or three thymine nucleotides. In other aspects, the at least one nucleotide is between one and four nucleotides, although the number of intervening nucleotides may differ depending on the sequence of the intervening nucleotides. In some aspects,

the oligonucleotide comprises SEQ ID NO:217, 218, 219, or 220. The nucleotides adjacent to a CpG—along with the CpG motif itself—constitute a CpG sequence element (e.g., XCGX, where X = any nucleotide). The oligonucleotides of the present disclosure, in some aspects comprise CpG sequence elements that are GCGA, GCGG, ACGC, CCGC, GCGT, TCGC, or any combination thereof.

[0069] In some embodiments of the present disclosures, the CpG motif comprises a CpG sequence element having four nucleotides. In some aspects, the oligonucleotide comprises at least two CpG sequence elements. In some aspects, the oligonucleotide comprises at least three CpG sequence elements. In some aspects, the oligonucleotide comprises at least four CpG sequence elements. In some aspects, the oligonucleotide comprises at least five CpG sequence elements. In some aspects, the oligonucleotide comprises at least six CpG sequence elements. In some aspects, the oligonucleotide comprises more than eight, ten, fifteen, or even twenty CpG sequence elements.

[0070] In other embodiments of the presently disclosed oligonucleotides, each of the CpG motifs are separated from every other CpG motif by a spacer or a combination of a spacer and at least one nucleotide. In some aspects, at least one CpG motif is separated from the nearest other CpG motif by a spacer or a combination of a spacer and at least one nucleotide, while at least two other CpG motifs are adjacent to each other. Although separated CpG motifs may enhance the immunostimulatory capabilities of the designed oligonucleotides, it is acknowledged that CpG motifs adjacent to each other can still stimulate TLR21.

[0071] The spacer employed to linearly separate CpG motifs can be any linkage that bridges at least a portion of the oligonucleotide between the CpG motifs. The spacer may be comprised of, but not necessarily limited to, a deoxyribose phosphate bridge, a multiple carbon chain, or a repeated chemical unit. One essential property of a spacer is the ability to form a chemical bond with the nucleotide backbone of the oligonucleotide. Therefore, in some embodiments the spacer is a deoxyribose phosphate bridge. The deoxyribose phosphate bridge may comprise nitrogenous bases in some aspects while in others the deoxyribose phosphate bridge is abasic. In some aspects, the oligonucleotide comprises SEQ ID NO:221, which comprises an abasic deoxyribose phosphate bridge.

[0072] In other embodiments of the present disclosure, the spacer comprises a carbon chain. The carbon chain can comprise two to twelve carbon atoms. Diols comprising a carbon chain can be used as the terminal alcohol groups can react with terminal alcohol and/or phosphate

groups of an oligonucleotide. In some embodiments, the carbon chain comprises two carbon atoms, and in some aspects, the carbon chain is derived from ethanediol. In some embodiments, the oligonucleotide comprises ODN-X2, wherein X2 is ethanediol.

[0073] Other embodiments of the present disclosure provide for the carbon chain comprising three carbon atoms. In some aspects of these embodiments, the carbon chain is derived from 1,3-propanediol. In some embodiments, the oligonucleotide comprises CG-Gw2X2, CG-Gw2X2-2, or ODN-X3, CG-Gw2X2-1, CG-Gw2X2-3, CG-Gw2X2-4, CG-Gw2X2-5, CG-G4T16X2-1, CG-G4T16X2-2, CG-G4T16X2-3, CG-G4T16X2-4, or CG-G4T16X2-5, wherein X2 is a three carbon chain; 2006-PDE5dG4-X2 wherein X2 is a three carbon chain derived from propanediol; or SEQ ID NO:250, wherein X4 is a three carbon chain derived from propanediol.

[0074] In yet other embodiments of the present disclosure the oligonucleotide comprises a carbon chain spacer, wherein the carbon chain comprises four carbon atoms. In some aspects of these embodiments, the carbon chain is derived from 1,4-butanediol. In some embodiments, the oligonucleotide comprises ODN-X4, wherein X4 is a four carbon chain derived from 1,4-butanediol.

[0075] In still other embodiments of the present disclosures, the oligonucleotide comprises a spacer having a repeated chemical unit. For example, in some embodiments, the repeated chemical unit is an ethylene glycol. The repeated chemical unit may be repeated two to twelve times. In some embodiments, ethylene glycol is repeated six times. Thus, in some aspects, the oligonucleotide comprises CCGC-Gw2X1, wherein X1 is a spacer derived from hexaethyleneglycol.

[0076] Although guanine nucleotide runs on the 3' terminus of an oligonucleotide results in little, if any, TLR21 stimulation, other nucleotide runs can impart enhanced immunogenicity to the oligonucleotide. Specifically, in some aspects of the present disclosures, the oligonucleotide may further comprise a tri-thymine nucleotide 3' terminal end. In some aspects, the oligonucleotide comprises SEQ ID NO:204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, or 215.

[0077] For each oligonucleotide disclosed herein, one skilled in the art would know that a nucleotide can be substituted with a nucleotide analog. The oligonucleotides in some embodiments comprise a phosphodiester backbone, although other embodiments of the oligonucleotides disclosed herein comprise a phosphorothioate backbone. Phosphorothioate backbones may, in some circumstances, be easier and more cost effective to manufacture.

[0078] In some embodiments of the present disclosure, the oligonucleotide may comprise a lipid moiety, which can lead to an increase in the oligonucleotide's immunogenicity. One possible

explanation for the increased immunogenicity is that the lipid moiety may function to enhance the bioavailability of the oligonucleotide. In some embodiments, the lipid moiety is at or near the 5' terminus of the oligonucleotide. This lipid "cap" may prevent degradation, increase solubility, improve the oligonucleotide's stability in a pharmaceutical composition, or any combination thereof. In some aspects, the lipid moiety is a cholesteryl.

[0079] The potency of the immunostimulatory oligonucleotide and the immunostimulatory composition can be characterized by their half-maximum effective concentration (EC_{50}), which is a measurement of the concentration necessary to induce a response that is half of the maximum response that can be attained by administering the composition. The lower the concentration, the more potent the oligonucleotide. In some aspects of the present disclosures, the immunostimulatory composition can have an EC_{50} in the pM range. In some aspects, the EC_{50} is between about 0.1 and 100 pM. In some aspects, the EC_{50} is between about 100 and 200 pM. In some aspects the EC_{50} is between about 200 and 300 pM. In some aspects, the EC_{50} is between about 300 and 400 pM. In some aspects the EC_{50} is between about 400 and 500 pM. In some aspects the EC_{50} is between about 500 and 600 pM. In some aspects the EC_{50} is between about 600 and 700 pM. In some aspects the EC_{50} is between about 700 and 800 pM. In some aspects the EC_{50} is between about 800 and 900 pM. In some aspects the EC_{50} is between about 900 and 1 nM. In still other aspects, the EC_{50} is less than about 100 pM.

[0080] Regarding the concentration of the oligonucleotide in the immunostimulatory composition, in some aspects the concentration of the oligonucleotide is between about 0.1 and 10 nM. In some aspects, the concentration of the oligonucleotide is between about 10 and 20 nM. In some aspects the concentration of the oligonucleotide is between about 20 and 30 nM. In some aspects, the concentration of the oligonucleotide is between about 30 and 40 nM. In some aspects the concentration of the oligonucleotide is between about 40 and 50 nM. In some aspects the concentration of the oligonucleotide is between about 50 and 60 nM. In some aspects the concentration of the oligonucleotide is between about 60 and 70 nM. In some aspects the concentration of the oligonucleotide is between about 70 and 80 nM. In some aspects the concentration of the oligonucleotide is between about 80 and 90 nM. In some aspects the concentration of the oligonucleotide is between about 90 and 100 nM. In still other aspects, the concentration of the oligonucleotide is less than about 20 nM.

[0081] The immunostimulatory composition may further comprise at least one additional oligonucleotide having a G-wire sequence in some embodiments of the present disclosure. Because the G-wire sequence facilitates the aggregation of other oligonucleotides having the same, or similar, G-wire sequence, one aspect of the immunostimulatory composition further comprises at least one additional oligonucleotide having a G-wire sequence. In some aspects in which the immunostimulatory composition comprises multiple oligonucleotides having G-wire sequences, the oligonucleotide and the at least one additional oligonucleotide have a G-wire conformation.

[0082] The ability of an oligonucleotide to stimulate TLR21 may be further enhanced according to some aspect of the invention by inserting additional CpG motifs. In some aspects, the at least one CpG motif is a plurality of CpG motifs, and the plurality of CpG motifs comprises two, three, four, or five CpG motifs. Distance between the CpG motifs can influence the oligonucleotide's TLR21 stimulatory properties. For this reason, some aspects of the disclosed oligonucleotides provide for insertion of at least one nucleotide or nucleotide analog between the CpG motifs. The at least one nucleotide may be two or three thymine nucleotides.

[0083] Other embodiments provide for inclusion of a spacer between each of the CpG motifs. The spacer must be able to bond to the 3' terminus of one adjacent nucleotide strand and to the 5' end of the other nucleotide strand. In some aspects, the spacer is a deoxyribose phosphate bridge, which can be abasic in some aspects.

[0084] The spacer, in some aspects, may comprise a carbon chain. In some embodiments the carbon chain comprises two carbon atoms. In some aspects the carbon chain is derived from ethanediol. Other embodiments provide for a carbon chain comprising three carbon atoms. In some aspects, the carbon chain is derived from 1,3-propanediol. In some embodiments, the carbon chain comprises four carbon atoms, and in some aspects the carbon chain is derived from 1,4-butanediol. In still other embodiments, the spacer comprises a repeated chemical unit. In some aspects, the repeated chemical unit is an ethylene glycol, and in some aspects the spacer is derived from hexaethyleneglycol.

[0085] Representative oligonucleotides of the present disclosure are identified in Table 1.

Table 1: Oligonucleotide sequences (lower case: PTO bonds, upper case PDE bonds)

5Chol-GCGT3-TG4T	SEQ ID NO:1	XTGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT X = 5'-Cholesteryl
2006-PTO	SEQ ID NO:3	tcgtcgttttgtcgttttgtcgtt

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE3dG1	SEQ ID NO:5	TCGTCGTTTTGTCGTTTTGTCGTTG
2006-PDE3dG2	SEQ ID NO:6	TCGTCGTTTTGTCGTTTTGTCGTTGG
2006-PDE3dG3	SEQ ID NO:7	TCGTCGTTTTGTCGTTTTGTCGTTGGG
2006-PDE3dG4	SEQ ID NO:8	TCGTCGTTTTGTCGTTTTGTCGTTGGGG
2006-PDE3dG5	SEQ ID NO:9	TCGTCGTTTTGTCGTTTTGTCGTTGGGGG
2006-PDE3dG6	SEQ ID NO:10	TCGTCGTTTTGTCGTTTTGTCGTTGGGGGG
2006-PDE3dG7	SEQ ID NO:11	TCGTCGTTTTGTCGTTTTGTCGTTGGGGGGG
2006-PDE3dG8	SEQ ID NO:12	TCGTCGTTTTGTCGTTTTGTCGTTGGGGGGGG
2006-PTO	SEQ ID NO:3	tcgtcgttttgtcgttttgtcgtt
2006-PDEV3	SEQ ID NO:13	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG1	SEQ ID NO:14	GTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG2	SEQ ID NO:15	GGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG3	SEQ ID NO:16	GGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4	SEQ ID NO:17	GGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG5	SEQ ID NO:18	GGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG7	SEQ ID NO:20	GGGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG8	SEQ ID NO:21	GGGGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dA6	SEQ ID NO:22	AAAAAATCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dC6	SEQ ID NO:23	CCCCCCTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dT6	SEQ ID NO:24	TTTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-Me	SEQ ID NO:25	GGGGGGTm5cGTm5cGTTTTGTm5cGTTTTGTm5cGTT m5c = 5-methyl-cytidine
2006-PDE5dC6-GC	SEQ ID NO:26	CCCCCCTGCTGCTTTTGTGCTTTTGTGCTT
2006-PDE5dT6-CA	SEQ ID NO:27	TTTTTTTCATCATTTTGTCATTTTGTCATT
2006-PTO3dG5	SEQ ID NO:28	tgggggtcgtcgttttgtcgttttgtcgtt
2006-PTO5dG6	SEQ ID NO:29	tcgtcgttttgtcgttttgtcgttgggggg

2006-PDE5dG6-A1	SEQ ID NO:30	AGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A2	SEQ ID NO:31	GAGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A3	SEQ ID NO:32	GGAGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A4	SEQ ID NO:33	GGGAGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A5	SEQ ID NO:34	GGGGAGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A6	SEQ ID NO:35	GGGGGATCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A12	SEQ ID NO:36	AAGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A23	SEQ ID NO:37	GAAGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A34	SEQ ID NO:38	GGAAGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A45	SEQ ID NO:39	GGGAAGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-A56	SEQ ID NO:40	GGGGAATCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C1	SEQ ID NO:41	CGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C2	SEQ ID NO:42	GCGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C3	SEQ ID NO:43	GGCGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C4	SEQ ID NO:44	GGGCGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-	SEQ ID NO:45	GGGGCGTCGTCGTTTTGTCGTTTTGTCGTT

C5		
2006-PDE5dG6-C6	SEQ ID NO:46	GGGGGCTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C12	SEQ ID NO:47	CCGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C23	SEQ ID NO:48	GCCGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C34	SEQ ID NO:49	GGCCGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C45	SEQ ID NO:50	GGGCCGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-C56	SEQ ID NO:51	GGGGCCTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T1	SEQ ID NO:52	TGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T2	SEQ ID NO:53	GTGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T3	SEQ ID NO:54	GGTGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T4	SEQ ID NO:55	GGGTGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T5	SEQ ID NO:56	GGGGTGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T6	SEQ ID NO:57	GGGGGTTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T12	SEQ ID NO:58	TTGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T23	SEQ ID NO:59	GTTGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T34	SEQ ID NO:60	GGTTGGTCGTCGTTTTGTCGTTTTGTCGTT

2006-PDE5dG6-T45	SEQ ID NO:61	GGGTTGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T56	SEQ ID NO:62	GGGGTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-A1	SEQ ID NO:63	AGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-A2	SEQ ID NO:64	GAGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-A3	SEQ ID NO:65	GGAGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-A4	SEQ ID NO:66	GGGATCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-C1	SEQ ID NO:67	CGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-C2	SEQ ID NO:68	GCGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-C3	SEQ ID NO:69	GGCGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-C4	SEQ ID NO:70	GGGCTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-T1	SEQ ID NO:71	TGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-T2	SEQ ID NO:72	GTGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-T3	SEQ ID NO:73	GGTGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4-T4	SEQ ID NO:74	GGGTTCGTCGTTTTGTCGTTTTGTCGTT
1668	SEQ ID NO:75	TCCATGACGTTCCTGATGCT
1668-3dG5	SEQ ID NO:76	TCCATGACGTTCCTGATGCTGGGGG
1668-5dG4	SEQ ID NO:77	GGGGTCCATGACGTTCCTGATGCT

1668-5dG6	SEQ ID NO:78	GGGGGGTCCATGACGTTCCTGATGCT
1826	SEQ ID NO:79	TCCATGACGTTCCTGACGTT
1826-3dG5	SEQ ID NO:80	TCCATGACGTTCCTGACGTTGGGGG
1826-5dG4	SEQ ID NO:81	GGGGTCCATGACGTTCCTGACGTT
1826-5dG6	SEQ ID NO:82	GGGGGGTCCATGACGTTCCTGACGTT
BW006	SEQ ID NO:83	TCGACGTTCGTCGTTCGTCGTTC
BW006-3dG5	SEQ ID NO:84	TCGACGTTCGTCGTTCGTCGTTCGGGGG
BW006-5dG4	SEQ ID NO:85	GGGGTCGACGTTCGTCGTTCGTCGTTC
BW006-5dG6	SEQ ID NO:86	GGGGGGTCGACGTTCGTCGTTCGTCGTTC
D-SLO1	SEQ ID NO:87	TCGCGACGTTCGCCCGACGTTCGGTA
D-SLO1-3dG5	SEQ ID NO:88	TCGCGACGTTCGCCCGACGTTCGGTAGGGGG
D-SLO1-5dG4	SEQ ID NO:89	GGGGTCGCGACGTTCGCCCGACGTTCGGTA
D-SLO1-5dG6	SEQ ID NO:90	GGGGGGTCGCGACGTTCGCCCGACGTTCGGTA
2395	SEQ ID NO:91	TCGTCGTTTTCGGCGCGCGCCG
2395-5dG4	SEQ ID NO:92	GGGGTCGTCGTTTTCGGCGCGCGCCG
2395-5dG6	SEQ ID NO:93	GGGGGGTCGTCGTTTTCGGCGCGCGCCG
M362	SEQ ID NO:94	TCGTCGTCGTTCGAACGACGTTGAT
M362-3dG5	SEQ ID NO:95	TCGTCGTCGTTCGAACGACGTTGATGGGGG
M362-5dG4	SEQ ID NO:96	GGGGTCGTCGTCGTTCGAACGACGTTGAT
M362-5dG6	SEQ ID NO:97	GGGGGGTCGTCGTCGTTCGAACGACGTTGAT
2007-PDE	SEQ ID NO:98	TCGTCGTTGTCGTTTTGTCGTT
2007-PDE3dG5	SEQ ID NO:99	TCGTCGTTGTCGTTTTGTCGTTGGGGG
2007-PDE5dG6	SEQ ID NO:100	GGGGGGTCGTCGTTGTCGTTTTGTCGTT
CPG-202	SEQ ID NO:101	GATCTCGCTCGCTCGCTAT
CPG-202-5dG6	EQ ID NO:102	GGGGGGGATCTCGCTCGCTCGCTAT
CPG-685	SEQ ID NO:103	TCGTCGACGTCGTTCGTTCTC
CPG-685-5dG6	SEQ ID NO:104	GGGGGGTCGTCGACGTCGTTCGTTCTC
CPG-2000	SEQ ID NO:105	TCCATGACGTTCCTGCAGTTCCTGACGTT
CPG-2000-5dG6	SEQ ID NO:106	GGGGGGTCCATGACGTTCCTGCAGTTCCTGACGTT
CPG-2002	SEQ ID NO:107	TCCACGACGTTTTCGACGTT

CPG-2002-5dG6	SEQ ID NO:108	GGGGGGTCCACGACGTTTTCGACGTT
2006-T4-PDE	SEQ ID NO:109	TTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-HuTel-1	SEQ ID NO:110	TTAGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-HuTel-2	SEQ ID NO:111	TTAGGGTTAGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Oxy1	SEQ ID NO:112	TTTTGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Oxy2	SEQ ID NO:113	GGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Oxy3	SEQ ID NO:114	GGGGTTTTGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-HuTel-1	SEQ ID NO:115	TTAGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-HuTel-2	SEQ ID NO:116	TTAGGGTTAGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-ScerTel	SEQ ID NO:117	TGTGGGTGTGTGTGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-cMyc	SEQ ID NO:118	GGAGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-cMyc2	SEQ ID NO:119	TGGAGGCTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-cMyc3	SEQ ID NO:120	TGGAGGCTGGAGGCTTTTTCGTCGTTTTGTCGTTTTGTCGTT
EA2-2006	SEQ ID NO:121	GCTGCGAGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT
EA2D-2006	SEQ ID NO:122	GCTGCGGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT
EA2a-2006	SEQ ID NO:123	CGAGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT
EA2aD-2006	SEQ ID NO:124	CGGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT
HCV-2006	SEQ ID NO:125	GGGCGTGGTGGGTGGGGTTCGTCGTTTTGTCGTTTTGTCGTT
HIV-93del-2006	SEQ ID NO:126	GGGGTGGGAGGAGGGTTCGTCGTTTTGTCGTTTTGTCGTT
Hema-2006	SEQ ID NO:127	GGGGTCGGGCGGGCCGGGTGTCGTCGTTTTGTCGTTTTGTCGTT
Insu-2006	SEQ ID NO:128	GGTGGTGGGGGGGGTTGGTAGGGTTCGTCGTTTTGTCGTTTTGTCGTT
IonK-2006	SEQ ID NO:129	GGGTTAGGGTTAGGGTAGGGTCGTCGTTTTGTCGTTTTGTCGTT
Scle-2006	SEQ ID NO:130	TGGGGGGGTGGGTGGGTTCGTCGTTTTGTCGTTTTGTCGTT
STAT-2006	SEQ ID NO:131	GGGCGGGCGGGCGGGCTCGTCGTTTTGTCGTTTTGTCGTT
TBA-2006	SEQ ID NO:132	GGTTGGTGTGGTTGGTCGTCGTTTTGTCGTTTTGTCGTT
TNF-2006	SEQ ID NO:133	GGTGGATGGCGCAGTCGGTCGTCGTTTTGTCGTTTTGTCGTT
apVEGF-D-2006	SEQ ID NO:134	TGGGGGTGGACGGGCCGGGTTCGTCGTTTTGTCGTTTTGT

		CGTT
apVEGF-2006	SEQ ID NO:135	TGTGGGGGTGGACGGGCCGGGTTCGTCGTTTTGTCGTTTTGTCGTT
HTR-2006	SEQ ID NO:136	GGGTTAGGGTTAGGGTTAGGGTCGTCGTTTTGTCGTTTTGTCGTT
bcl-2-2006	SEQ ID NO:137	GGGCGCGGGAGGAAGGGGGCGGGTCGTCGTTTTGTCGTTTTGTCGTT
c-myc-2006	SEQ ID NO:138	AGGGTGGGGAGGGTGGGGATCGTCGTTTTGTCGTTTTGTCGTT
c-kit87-2006	SEQ ID NO:139	AGGGAGGGCGCTGGGAGGAGGGTCGTCGTTTTGTCGTTTTGTCGTT
vegf-2006	SEQ ID NO:140	GGGGCGGGCCGGGGGCGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Gwire1	SEQ ID NO:141	GGGGTTGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Gwire2	SEQ ID NO:142	GGGGTTGGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006PDE5dG4-T1-6	SEQ ID NO:143	TGGGGTTCGTCGTTTTGTCGTTTTGTCGTT
1-ACGA	SEQ ID NO:144	TTTTTTTACGATTT
2-GCGA	SEQ ID NO:145	TTTTTTTGCGATTT
3-CCGA	SEQ ID NO:146	TTTTTTTCCGATTT
4-TCGA	SEQ ID NO:147	TTTTTTTTCGATTT
5-ACGG	SEQ ID NO:148	TTTTTTTACGGTTT
6-GCGG	SEQ ID NO:149	TTTTTTTGCGGTTT
7-CCGG	SEQ ID NO:150	TTTTTTTCCGGTTT
8-TCGG	SEQ ID NO:151	TTTTTTTTCGGTTT
9-ACGC	SEQ ID NO:152	TTTTTTTACGCTTT
10-GCGC	SEQ ID NO:153	TTTTTTTGCGCTTT
11-CCGC	SEQ ID NO:154	TTTTTTTCCGCTTT
12-TCGC	SEQ ID NO:155	TTTTTTTTCGCTTT
13-ACGT	SEQ ID NO:156	TTTTTTTACGTTTT
14-GCGT	SEQ ID NO:157	TTTTTTTGCGTTTT
15-CCGT	SEQ ID NO:158	TTTTTTTCCGTTTT
16-TCGT	SEQ ID NO:159	TTTTTTTTCGTTTT
17-ACGA-5dG6	SEQ ID NO:160	GGGGGGTTTTTTTACGATTT
18-GCGA-5dG6	SEQ ID NO:161	GGGGGGTTTTTTTGCGATTT
19-CCGA-5dG6	SEQ ID NO:162	GGGGGGTTTTTTTCCGATTT
20-TCGA-5dG6	SEQ ID NO:163	GGGGGGTTTTTTTTCGATTT
21-ACGG-5dG6	SEQ ID NO:164	GGGGGGTTTTTTTACGGTTT
22-GCGG-5dG6	SEQ ID NO:165	GGGGGGTTTTTTTGCGGTTT
23-CCGG-5dG6	SEQ ID NO:166	GGGGGGTTTTTTTCCGGTTT
24-TCGG-5dG6	SEQ ID NO:167	GGGGGGTTTTTTTTCGGTTT
25-ACGC-5dG6	SEQ ID NO:168	GGGGGGTTTTTTTACGCTTT

26-GCGC-5dG6	SEQ ID NO:169	GGGGGGTTTTTTTGCGCTTT
27-CCGC-5dG6	SEQ ID NO:170	GGGGGGTTTTTTTCCGCTTT
28-TCGC-5dG6	SEQ ID NO:171	GGGGGGTTTTTTTTCGCTTT
29-ACGT-5dG6	SEQ ID NO:172	GGGGGGTTTTTTTACGTTTT
30-GCGT-5dG6	SEQ ID NO:173	GGGGGGTTTTTTTGCGTTTT
31-CCGT-5dG6	SEQ ID NO:174	GGGGGGTTTTTTTCCGTTTT
32-TCGT-5dG6	SEQ ID NO:175	GGGGGGTTTTTTTTCGTTTT
33-ACGA-Gwire2	SEQ ID NO:176	GGGGTTGGGGTTTTTTTTTTTACGATTT
34-GCGA-Gwire2	SEQ ID NO:177	GGGGTTGGGGTTTTTTTTTTTGCGATTT
35-CCGA-Gwire2	SEQ ID NO:178	GGGGTTGGGGTTTTTTTTTTTCCGATTT
36-TCGA-Gwire2	SEQ ID NO:179	GGGGTTGGGGTTTTTTTTTTTCGATTT
37-ACGG-Gwire2	SEQ ID NO:180	GGGGTTGGGGTTTTTTTTTTTACGGTTT
38-GCGG-Gwire2	SEQ ID NO:181	GGGGTTGGGGTTTTTTTTTTTGCGGTTT
39-CCGG-Gwire2	SEQ ID NO:182	GGGGTTGGGGTTTTTTTTTTTCCGGTTT
40-TCGG-Gwire2	SEQ ID NO:183	GGGGTTGGGGTTTTTTTTTTTCGGTTT
41-ACGC-Gwire2	SEQ ID NO:184	GGGGTTGGGGTTTTTTTTTTTACGCTTT
42-GCGC-Gwire2	SEQ ID NO:185	GGGGTTGGGGTTTTTTTTTTTGCGCTTT
43-CCGC-Gwire2	SEQ ID NO:186	GGGGTTGGGGTTTTTTTTTTTCCGCTTT
44-TCGC-Gwire2	SEQ ID NO:187	GGGGTTGGGGTTTTTTTTTTTCGCTTT
45-ACGT-Gwire2	SEQ ID NO:188	GGGGTTGGGGTTTTTTTTTTTACGTTTT
46-GCGT-Gwire2	SEQ ID NO:189	GGGGTTGGGGTTTTTTTTTTTGCGTTTT
47-CCGT-Gwire2	SEQ ID NO:190	GGGGTTGGGGTTTTTTTTTTTCCGTTTT
48-TCGT-Gwire2	SEQ ID NO:191	GGGGTTGGGGTTTTTTTTTTTCGTTTT
GCGT-Gwire2-GC	SEQ ID NO:192	GGGGTTGGGGTTTTTTTTTTGGCTTTT
GCGT-Gwire2-TG	SEQ ID NO:193	GGGGTTGGGGTTTTTTTTTTGTGTTTT
GCGT-Gwire2-CA	SEQ ID NO:194	GGGGTTGGGGTTTTTTTTTTGCATTTT
GCGT-Gwire2-T1	SEQ ID NO:195	GGGGTTGGGGTTTTTTTTTGCGTTTT
GCGT-Gwire2-T2	SEQ ID NO:196	GGGGTTGGGGTTTTTTTTGCGTTTT
GCGT-Gwire2-T3	SEQ ID NO:197	GGGGTTGGGGTTTTTTTGCGTTTT
GCGT-Gwire2-T4	SEQ ID NO:198	GGGGTTGGGGTTTTTTGCGTTTT
GCGT-Gwire2-T5	SEQ ID NO:199	GGGGTTGGGGTTTTTGCGTTTT
GCGT-Gwire2-T6	SEQ ID NO:200	GGGGTTGGGGTTTTGCGTTTT
GCGT-Gwire2-eT1	SEQ ID NO:201	GGGGTTGGGGTTTTTTTTTTGCGTTT
GCGT-Gwire2-eT2	SEQ ID NO:202	GGGGTTGGGGTTTTTTTTTTGCGTT
GCGT-Gwire2-eT3	SEQ ID NO:203	GGGGTTGGGGTTTTTTTTTTGCGT
GCGT-Gwire3	SEQ ID NO:224	GGGGTTGGGGTTGGGGTTTTTTTTTTGCGTTTT
GCGT-Gwire2-do	SEQ ID NO:204	GGGGTTGGGGTTTTTTTTTTGCGTTTTGCGTTTT
GCGT-Gwire2-tri	SEQ ID NO:205	GGGGTTGGGGTTTTTTTTTTGCGTTTTGCGTTTTTGCGTTTT
GCGA-Gwire2	SEQ ID NO:177	GGGGTTGGGGTTTTTTTTTTGCGATTT

GCGA-Gwire2-do	SEQ ID NO:206	GGGGTTGGGGTTTTTTTTTTGCGATTTGCGATTT
GCGA-Gwire2-tri	SEQ ID NO:207	GGGGTTGGGGTTTTTTTTTTGCGATTTGCGATTTGCGATTT
ACGC-Gwire2	SEQ ID NO:184	GGGGTTGGGGTTTTTTTTTTACGCTTT
ACGC-Gwire2-do	SEQ ID NO:208	GGGGTTGGGGTTTTTTTTTTACGCTTTACGCTTT
ACGC-Gwire2-tri	SEQ ID NO:209	GGGGTTGGGGTTTTTTTTTTACGCTTTACGCTTTACGCTTT
TCGC-Gwire2	SEQ ID NO:187	GGGGTTGGGGTTTTTTTTTTCGCTTT
TCGC-Gwire2-do	SEQ ID NO:210	GGGGTTGGGGTTTTTTTTTTCGCTTTTCGCTTT
TCGC-Gwire2-tri	SEQ ID NO:211	GGGGTTGGGGTTTTTTTTTTCGCTTTTCGCTTTTCGCTTT
CCGC-Gwire2	SEQ ID NO:186	GGGGTTGGGGTTTTTTTTTTCCGCTTT
CCGC-Gwire2-do	SEQ ID NO:212	GGGGTTGGGGTTTTTTTTTTCCGCTTTCCGCTTT
CCGC-Gwire2-tri	SEQ ID NO:213	GGGGTTGGGGTTTTTTTTTTCCGCTTTCCGCTTTCCGCTTT
GCGG-Gwire2-mo	SEQ ID NO:181	GGGGTTGGGGTTTTTTTTTTGCGGTTT
GCGG-Gwire2-do	SEQ ID NO:214	GGGGTTGGGGTTTTTTTTTTGCGGTTTGCGGTTT
GCGG-Gwire2-tri	SEQ ID NO:215	GGGGTTGGGGTTTTTTTTTTGCGGTTTGCGGTTTGCGGTTT
CG-Gw2-T0	SEQ ID NO:216	GGGGTTGGGGTTTTTTTCGCGCGTTT
CG-Gw2-T1	SEQ ID NO:217	GGGGTTGGGGTTTTTTTCGTCGTCGTTT
CG-Gw2-T2	SEQ ID NO:218	GGGGTTGGGGTTTTTTTCGTTCGTTCGTTT
CG-Gw2-T3	SEQ ID NO:219	GGGGTTGGGGTTTTTTTCGTTTCGTTTCGTTT
CG-Gw2-T4	SEQ ID NO:220	GGGGTTGGGGTTTTTTTCGTTTTCGTTTTCGTTT
CG-Gw2-abase	SEQ ID NO:221	GGGGTTGGGGTTTTTTTCGXCGXCGTTT X = abasic site
CG-Gw2X1	SEQ ID NO:222**	GGGGTTGGGGTTTTTTTCGX1CGX1CGTTT X1=C18
CG-Gw2X2	SEQ ID NO:223**	GGGGTTGGGGTTTTTTTCGX2CGX2CGTTT X2=C3
CG-Gw2X2-1	SEQ ID NO:225**	GGGGTTGGGGTTTTTTTCGX2CGTTT X2=C3
CG-Gw2X2-2	SEQ ID NO:223**	GGGGTTGGGGTTTTTTTCGX2CGX2CGTTT X2=C3
CG-Gw2X2-3	SEQ ID NO:226**	GGGGTTGGGGTTTTTTTCGX2CGX2CGX2CGTTT X2=C3
CG-Gw2X2-4	SEQ ID NO:227**	GGGGTTGGGGTTTTTTTCGX2CGX2CGX2CGX2CGTTT X2=C3
CG-Gw2X2-5	SEQ ID NO:228**	GGGGTTGGGGTTTTTTTCGX2CGX2CGX2CGX2CGX2CGTTT X2=C3
CG-G4T16X2-1	SEQ ID NO:229**	TGGGGTTTTTTTTCGX2CGTTT X2=C3
CG-G4T16X2-2	SEQ ID NO:230**	TGGGGTTTTTTTTCGX2CGX2CGTTT X2=C3
CG-G4T16X2-3	SEQ ID	TGGGGTTTTTTTTCGX2CGX2CGX2CGTTT

	NO:231**	X2=C3
CG-G4T16X2-4	SEQ ID NO:232**	TGGGGTTTTTTTTCGX2CGX2CGX2CGX2CGTTT X2=C3
CG-G4T16X2-5	SEQ ID NO:233**	TGGGGTTTTTTTTCGX2CGX2CGX2CGX2CGX2CGTTT X2=C3
ODN-X2	SEQ ID NO:234**	GGGGTTGGGGTTTTTTTTCGX2CGX2CGTTT (X2 = Ethanediol)
ODN-X3	SEQ ID NO:223**	GGGGTTGGGGTTTTTTTTCGX3CGX3CGTTT (X3 = Propanediol)
ODN-X4	SEQ ID NO:235**	GGGGTTGGGGTTTTTTTTCGX4CGX4CGTTT (X4 = Butanediol)
ODN-X6	SEQ ID NO:236**	GGGGTTGGGGTTTTTTTTCGX6CGX6CGTTT (X6 = Hexanediol
ODN-X9	SEQ ID NO:237**	GGGGTTGGGGTTTTTTTTCGX9CGX9CGTTT (X9 = Nonanediol)
ODN-X12	SEQ ID NO:238**	GGGGTTGGGGTTTTTTTTCGX12CGX12CGTTT (X12 = Dodecanediol)
ODN-Xab	SEQ ID NO:239	GGGGTTGGGGTTTTTTTTCGXabCGXabCGTTT (Xab = dSpacer (abasic))
ODN-XtrEG	SEQ ID NO:240**	GGGGTTGGGGTTTTTTTTCGXtrCGXtrCGTTT (Xtr = Triethyleneglycol)
ACGC-Gw2X1	SEQ ID NO:241**	GGGGTTGGGGTTTTTTTTACGCX1ACGCX1ACGCTTT X1 = C18 (HEG*)
CCGC-Gw2X1	SEQ ID NO:242**	GGGGTTGGGGTTTTTTTTCCGCX1CCGCX1CCGCTTT X1 = C18 (HEG*)
ACGC-Gw2X2	SEQ ID NO:243**	GGGGTTGGGGTTTTTTTTACGCX2ACGCX2ACGCTTT X2 = Propanediol
CCGC-Gw2X2	SEQ ID NO:244**	GGGGTTGGGGTTTTTTTTCCGCX2CCGCX2CCGCTTT X2 = Propanediol
ACGC-G4T16-X2	SEQ ID NO:245**	TGGGGTTTTTTTTACGCX2ACGCX2ACGCTTT X2 = Propanediol
CCGC-G4T16-X2	SEQ ID NO:246**	TGGGGTTTTTTTTCCGCX2CCGCX2CCGCTTT X2 = Propanediol
2006-PDE5dG4-X1	SEQ ID NO:247**	GGGGX1TCGTCGTTTTGTCGTTTTGTCGTT X1 = C18 (HEG*)
2006-PDE5dG4-X2	SEQ ID NO:248**	GGGGX2TCGTCGTTTTGTCGTTTTGTCGTT X2 = Propanediol
2006-PDE5dG4-X3	SEQ ID NO:249**	GGGGX3GGGGTCGTCGTTTTGTCGTTTTGTCGTT X3 = C18 (HEG*)
2006-PDE5dG4-X4	SEQ ID NO:250**	GGGGX4GGGGTCGTCGTTTTGTCGTTTTGTCGTT X4 = Propanediol
2006-T4-5dTG4T	SEQ ID NO:251	TGGGGTTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4TG4T-3C	SEQ ID NO:251	TGGGGTTTTTTCGTCGTTTTGTCGTTTTGTCGTTX 3′-Cholesteryl
5Chol-GCGT3-TG4T	SEQ ID NO:1	XTGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT X = 5′-Cholesteryl

GCGT3-TG4T	SEQ ID NO:252	TGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT
GCGT-3-Gw2-5Chol	SEQ ID NO:253	XGGGGTTGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT 5′-Cholesteryl
GCGT-3-Gw2	SEQ ID NO:253	GGGGTTGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT
GCGT3-5Chol	SEQ ID NO:254	XTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT X = 5′-Cholesteryl
GCGT3	SEQ ID NO:254	TTTTTTTGCGTTTTTGCGTTTTTGCGTTTT
5Chol-CCGC3-Gw2	SEQ ID NO:255	XGGGGTTGGGGTTTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT X = 5′-Cholesteryl
CCGC3-Gw2	SEQ ID NO:255	GGGGTTGGGGTTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT
5Chol-CCGC3	SEQ ID NO:256	XTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT X = 5′-Cholesteryl
CCGC3	SEQ ID NO:256	TTTTTTTCCGCTTTTCCGCTTTTCCGCTTT
*Hexaethyleneglycol **As referred to herein, sequence names (e.g., "CG-Gw2X1," etc.) refer to the full sequences shown in this table, including the X1, X2, X3, X4, X6, X9, X12, and Xtr non-nucleotide linkers.		

[0086] The immunogenic nucleic acid plasmids described herein are enriched in CpG motifs. In some aspects, the immunogenic nucleic acid plasmids contain more than 20% CpG motifs compared to the frequency of CpG motifs found in vertebrate nucleic acid sequences.

[0087] In some aspects, the present disclosure relates to immunogenic nucleic acid plasmids that do not comprise an antibiotic resistance gene. In some aspects, the plasmids do not comprise a nucleic acid sequence coding for a full-length or functional selectable or screenable marker. For example, the pGCMB75.6 plasmid described herein does not comprise any full-length or functional selectable or screenable marker genes. The sequence of pGCMB75.6 is provided in SEQ ID NO:265 (Table 1A). In some aspects, the plasmids described herein do not encode an immunogen.

[0088] In some aspects, the immunogenic plasmids may comprise a nucleic acid sequence coding for a selectable or screenable marker gene that is not an antibiotic resistance gene. For example, the pLacZMB75.6 plasmid described herein comprises a LacZ gene as a screenable marker. The sequence of pLacZMB75.6 is provided in SEQ ID NO:268. In still other aspects, the plasmid will contain an antibiotic resistance gene. For example, pMB75.6 comprises a nucleic acid

sequence encoding a resistance to the antibiotic kanamycin. The sequence of pMB75.6 is provided in SEQ ID NO:266.

[0089] It will be appreciated that the nucleotide sequence of the pMB75.6, pGCMB75.6, or pLacZMB75.6 plasmid may be varied to a certain extent without significantly adversely affecting its immunostimulatory properties. In some aspects are provided an immunogenic nucleic acid plasmids comprising or consisting of a nucleic acid sequence having at least 89% sequence identity with the sequence of pGCMB75.6 (SEQ ID NO: 265). In some aspects, the immunogenic plasmid comprises a nucleic acid sequence having at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the sequence of pGCMB75.6 (SEQ ID NO:265). In some aspects, the immunogenic nucleic acid plasmid comprises the sequence of pGCMB75.6 (SEQ ID NO:265).

[0090] In some aspects are provided immunogenic nucleic acid plasmids comprising a nucleic acid sequence having at least 84% sequence identity with the sequence of pLacZMB75.6 (SEQ ID NO:268). In some aspects, the immunogenic plasmid comprises or consists of a nucleic acid sequence having at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the sequence of pLacZMB75.6 (SEQ ID NO:268). In some aspects, the immunogenic nucleic acid comprises a plasmid having the sequence of pLacZMB75.6 (SEQ ID NO:268).

[0091] In some aspects are provided immunogenic nucleic acid plasmids comprising a nucleic acid sequence having at least 80% sequence identity with the sequence of SEQ ID NO:266. In some aspects, the immunogenic plasmid comprises or consists of a nucleic acid sequence having at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the sequence of SEQ ID NO:266. In some aspects, the immunogenic nucleic acid plasmid comprises the sequence of SEQ ID NO:266.

[0092] In some aspects are provided an immunogenic nucleic acid plasmid comprising a nucleic acid sequence having at least 80% sequence identity with the sequence of pMB75.6_AscI (SEQ ID NO:267). In some aspects, the immunogenic plasmid comprises or consists of a nucleic acid sequence having at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the sequence of SEQ ID NO:267. In some aspects, the immunogenic nucleic acid plasmid comprises the sequence of SEQ ID NO:267.

Table 1A: Plasmid sequences

pGCMB75.6 (SEQ ID NO:265)	
tgaccgccca acgacccccg cccattgacg tcaataatga cgtatgttcc catagtaacg	60
ccaataggga ctttccattg acgtcaatgg gtggagtatt tacggtaaac tgcccacttg	120
gcagtacatc aagtgtatca tatgccaagt ccgcccccta ttgacgtcaa tgacggtaaa	180
tggcccgcct ggcattatgc ccagtacatg accttacggg actttcctac ttggcagtac	240
atctacgtat tagtcatcgc tattaccatg gtgatgcggt tttggcagta catcaatggg	300
cgtggatagc ggtttgactc acggggattt ccaagtctcc accccattga cgtcaatggg	360
agtttgtttt ggcaccaaaa tcaacgggac tttccaaaat gtcgtaacaa ctccgcccca	420
ttgacgcaaa tgggcggtag gcgtgtacgg tgggaggtct atataagcag agctcgttta	480
gtgaaccgtc agatcgcctg gagacgccat ccacgctgtt ttgacctcca tagaagacac	540
cgggaccgat ccagcctccc ctcgaagccg atctgataac ggtaccgata agctggcggc	600
cgattaagct acagaagttg gtcgtgaggc actgggcagg taagtatcaa ggttacaaga	660
caggtttaag gagaccaata gaaactgggc ttgtcgagac agagaagact cttgcgtttc	720
tgataggcac ctattggtct tactgacatc cactttgcct ttctctccac aggtgtccac	780
tcccaggttc aattacagct cttaagcagc cgcaagcttg atatcgaatt cctgcagccc	840
gggggatcca ctagttctag agcggccgcc accgcggtgg agctcgaatt atcagatcga	900
ttaataacta tgctcaaaaa ttgtgtacct ttagcttttt aatttgtaaa ggggttaata	960
aggaatattt gatgtatagt gccttgacta gagatcataa tcagccatac cacatttgta	1020
gaggttttac ttgctttaaa aaacctccca cacctccccc tgaacctgaa acataaaatg	1080
aatgcaattg ttgttgttaa cttgtttatt gcagcttata atggttacaa ataaagcaat	1140
agcatcacaa atttcacaaa taaagcattt ttttcactgc attctagttg tggtttgtcc	1200
aaactcatca atgtatctta tcatgtctgg atcatcagat ctgccggtct ccctatagtg	1260
agtcgtatta atttcgataa gccaggttaa cctgcattaa tgaatcggcc aacgcgcggg	1320

gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg ctcactgact cgctgcgctc 1380
ggtcgttcgg ctgcggcgag cggtatcagc tcactcaaag gcggtaatac ggttatccac 1440
agaatcaggg gataacgcag gaaagaacat gtgagcaaaa ggccagcaaa aggccaggaa 1500
ccgtaaaaag gccgcgttgc tggcgttttt ccataggctc cgcccccctg acgagcatca 1560
caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa gataccaggc 1620
gtttccccct ggaagctccc tcgtgcgctc tcctgttccg accctgccgc ttaccggata 1680
cctgtccgcc tttctccctt cgggaagcgt ggcgctttct catagctcac gctgtaggta 1740
tctcagttcg gtgtaggtcg ttcgctccaa gctgggctgt gtgcacgaac cccccgttca 1800
gcccgaccgc tgcgccttat ccggtaacta tcgtcttgag tccaacccgg taagacacga 1860
cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt atgtaggcgg 1920
tgctacagag ttcttgaagt ggtggcctaa ctacggctac actagaagaa cagtatttgg 1980
tatctgcgct ctgctgaagc cagttacctt cggaaaaaga gttggtagct cttgatccgg 2040
caaacaaacc accgctggta gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag 2100
aaaaaaagga tctcaagaag atcctttgat cttttctacg gggtctgacg ctcagtggaa 2160
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caagagacag gatgaggatc gtttcgcagc ttttcattct gactgcaacg ggcaataagt 2280
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gatatcatca gggcagaccg gttacatccc cctaacaagc tgtataaaga gaaatactat 2460
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tttgaatgta tttagaaaaa taaacaaata ggggttccgc gcacatttcc ccgaaaagtg 3300
ccacctaaat tgtaagcgtt aatattttgt taaaattcgc gttaaatttt tgttaaatca 3360
gctcattttt taaccaatag gccgaaatcg gcaaaatccc ttataaatca aaagaataga 3420
ccgagatagg gttgagtgtt gttccagttt ggaacaagag tccactatta aagaacgtgg 3480
actccaacgt caaagggcga aaaaccgtct atcagggcga tggcccacta cgtgaaccat 3540
caccctaatc aagttttttg gggtcgaggt gccgtaaagc actaaatcgg aaccctaaag 3600

ggagcccccg atttagagct tgacggggaa agccggcgaa cgtggcgaga aaggaaggga	3660
agaaagcgaa aggagcgggc gctagggcgc tggcaagtgt agcggtcacg ctgcgcgtaa	3720
ccaccacacc cgccgcgctt aatgcgccgc tacagggcgc gtcccattcg ccattcaggc	3780
tgcgcaactg ttgggaaggg cgatcggtgc gggcctcttc gctattacgc cagctggcga	3840
aaggggggatg tgctgcaagg cgattaagtt gggtaacgcc agggttttcc cagtcacgac	3900
gttgtaaaac gacggccagt gagcgcgcgt aatacgactc actatagggc gaattgggta	3960
ccgggccccc cctcgagcag gatctataca ttgaatcaat attggcaatt agccatatta	4020
gtcattggtt atatagcata aatcaatatt ggctattggc cattgcatac gttgtatcta	4080
tatcataata tgtacattta tattggctca tgtccaatat gaccgccatg ttgacattga	4140
ttattgacta gttattaata gtaatcaatt acggggtcat tagttcatag cccatatatg	4200
gagttccgcg ttacataact tacggtaaat ggcccgcctg gc	4242
pMB75.6 (SEQ ID NO:266)	
ctaaattgta agcgttaata ttttgttaaa attcgcgtta aatttttgtt aaatcagctc	60
atttttaac caataggccg aaatcggcaa aatcccttat aaatcaaaag aatagaccga	120
gatagggttg agtgttgttc agtttggaa caagagtcca ctattaaaga acgtggactc	180
caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc	240
ctaatcaagt tttttggggt cgaggtgccg taaagcacta aatcggaacc ctaaagggag	300
cccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aagggaagaa	360
agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac	420
cacacccgcc gcgcttaatg cgccgctaca gggcgcgtcc cattcgccat tcaggctgcg	480
caactgttgg gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg	540
gggatgtgct gcaaggcgat taagttgggt aacgccaggg ttttcccagt cacgacgttg	600
taaaacgacg gccagtgagc gcgcgtaata cgactcacta tagggcgaat tgggtaccgg	660
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[0093] Further provided herein are immunogenic nucleic acids or immunogenic plasmids capable of stimulating an immune response including nucleic acid sequences that hybridize under high stringency conditions to SEQ ID NO:265, SEQ ID NO:266, SEQ ID NO:267, or SEQ ID NO:268. Suitable nucleic acid sequences include those that are homologous, substantially similar, or identical to the nucleic acids described herein. In some aspects, homologous nucleic acid sequences will have a percent identity of at least about 75%, 76%, 77%, 78%, 79%, 80% 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to SEQ ID NO:265 or the respective complementary sequence. In other aspects, homologous nucleic acid sequences will have a sequence similarity of at least about 75%, 76%, 77%, 78%, 79%, 80% 81%, 82%, 83%, 84%, 85%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to SEQ ID NO:268 or the respective complementary sequence. In other aspects, homologous nucleic acid sequences will have a sequence similarity of at least about 75%, 76%, 77%, 78%, 79%, 80% 81%, 82%, 83%, 84%, 85%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to SEQ ID NO:266 or the respective complementary sequence. In other aspects, homologous nucleic acid sequences will have a sequence similarity of at least about 75%, 76%, 77%, 78%, 79%, 80% 81%, 82%, 83%, 84%, 85%, 86%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to SEQ ID NO:267 or the respective complementary sequence. Sequence similarity may be calculated using a number of algorithms known in the art, such as BLAST, described in Altschul, S. F., et al., J. Mol. Biol. 215:403-10, 1990. The nucleic acids may differ in sequence from the above-described nucleic acids due to the degeneracy of the genetic code. In general, a reference sequence will be 18 nucleotides, more usually 30 or more nucleotides, and may comprise the entire nucleic acid sequence of the composition for comparison purposes.

[0094] Nucleic acids that can hybridize to SEQ ID NO:265, SEQ ID NO:266, SEQ ID NO:267, or SEQ ID NO:268 are contemplated herein. Stringent hybridization conditions include conditions such as hybridization at 50°C or higher and 0.1X SSC (15 mM sodium chloride/1.5 mM

sodium citrate). Another example is overnight incubation at 42°C in a solution of 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA, followed by washing in 0.1X SSC at about 65°C. Exemplary stringent hybridization conditions are hybridization conditions that are at least about 80%, 85%, 90%, or 95% as stringent as the above specific conditions. Other stringent hybridization conditions are known in the art and may also be employed to identify homologs of the nucleic acids of the present disclosure (Current Protocols in Molecular Biology, Unit 6, pub. John Wiley & Sons, N.Y. 1989).

[0095] It will be appreciated that the nucleotide sequences of the immunogenic nucleic acid plasmids may be varied to a certain extent without significantly adversely affecting their immunogenic properties. The nucleic acid sequence of such a variant nucleic acid plasmid molecule will usually differ by one or more nucleotides. The sequence changes may be substitutions, insertions, deletions, or a combination thereof. Techniques for mutagenesis of cloned genes are known in the art. Methods for site specific mutagenesis may be found in Gustin et al., Biotechniques 14:22, 1993; Barany, Gene 37:111-23, 1985; Colicelli et al., Mol. Gen. Genet. 199:537-9, 1985; and Sambrook et al., Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp. 15.3-15.108 and all incorporated herein by reference. In summary, the invention relates to nucleic acid plasmid molecules, and variants or mutants thereof, capable of stimulating an innate immune response in a subject. Also, the invention encompasses the intermediary RNAs encoded by the described nucleic acids, as well as any resultant amino acid sequences encoded by the nucleic acid plasmids described herein.

[0096] In some aspects, where the nucleotide sequence of the immunogenic nucleic acid plasmid varies from the sequence provided in SEQ ID NOs:265, 266, 267, or 268, the CpG dinucleotides in the immunogenic nucleic acid plasmid are preferably left intact. Alternatively, if the nucleotide sequence of the immunogenic plasmid is altered such that a CpG dinucleotide is eliminated, the sequence of the immunogenic nucleic acid plasmid may be altered at another location such that the total number of CpG dinucleotides in the nucleic acid plasmid remains the same. Further CpG dinucleotides in addition to those already present in the immunogenic nucleic acid plasmid may also be introduced. Thus, for example, the immunogenic nucleic acid plasmids described herein comprise at least about 200, at least about 220, at least about 240, at least about 260, at least about 270, at least about 275, at least about 280, at least about 283, at least about 285,

or at least about 288 CpG dinucleotides. In some embodiments, the immunogenic nucleic acid plasmid can comprise 283 CpG dinucleotides. In some embodiments, CpG dinucleotides in addition to those already present in the nucleotide sequences of pGCMB75.6 or pLacZMB75.6 are introduced into the plasmid.

[0097] In some aspects, where the nucleotide sequence of the immunogenic nucleic acid plasmid varies from the sequences provided herein, the CpG motif types in the immunogenic nucleic acid are varied to modulate the resultant activation of a cytosolic nucleic acid surveillance molecule, i.e., TLR21 and/or TLR9. For example, the number of immune stimulatory CpG motifs may be increased to enhance the activation of at least one cytosolic nucleic acid surveillance molecule responsive to an immunogenic nucleic acid plasmid. Alternatively, the number of non-immune stimulatory CpG motifs may be increased to reduce the activation of at least one cytosolic nucleic acid surveillance molecule. In some aspects, the number of stimulatory and nonstimulatory CpG motifs can be modified to enhance the activation of at least one cytosolic nucleic acid surveillance molecule and reduce the activation of at least one cytosolic nucleic acid surveillance molecule.

[0098] A suitable immunogenic nucleic acid plasmid molecule includes any of the immunogenic coding and noncoding nucleic acids described herein. Coding nucleic acid sequences encode at least a portion of a protein or peptide, while non-coding sequence does not encode any portion of a protein or peptide. According to the present invention, "non-coding" nucleic acids can include regulatory regions of a transcription unit, such as a promoter region. The term, "empty vector" can be used interchangeably with the term "non-coding," and particularly refers to a nucleic acid sequence in the absence of a protein coding portion, such as a plasmid vector without a gene insert. Expression of a protein encoded by the nucleic acid plasmids described herein is not required for inducing an immune response; therefore, the plasmids need not contain any coding sequences operatively linked to a transcription control sequence. However, further advantages may be obtained (i.e., antigen-specific and enhanced immunity) by including in the immunomodulatory composition at least one nucleic acid sequence (DNA or RNA) which encodes an immunogen and/or a cytokine. Such a nucleic acid sequence encoding an immunogen and/or a cytokine may be included in the immunogenic nucleic acid plasmids described herein, or may be included in a separate nucleic acid (e.g., a separate plasmid) in the composition.

[0099] In some embodiments of the immunomodulatory compositions described herein, the immunomodulatory composition comprises a liposomal delivery vehicle and at least one of the

immunogenic nucleic acid plasmids described herein. Suitable immunomodulatory compositions are described in U.S. Patent Application Publications Nos. 2012/0064151 A1 and 2013/0295167 A1, the contents of both are hereby incorporated by reference in their entirety.

[0100] A suitable liposomal delivery vehicle comprises a lipid composition that is capable of delivering nucleic acid molecules to the tissues of a treated subject. In some embodiments, a liposomal delivery vehicle may be capable of remaining stable in a subject for a sufficient amount of time to deliver a nucleic acid molecule and/or a biological agent. For example, the liposomal delivery vehicle is stable in the recipient subject for at least about five minutes, for at least about 1 hour, or for at least about 24 hours.

[0101] A liposomal delivery vehicle as described herein comprises a lipid composition that is capable of fusing with the plasma membrane of a cell to deliver a nucleic acid molecule into a cell. When the nucleic acid molecule encodes one or more proteins, the nucleic acid:liposome complex has, in some aspects, a transfection efficiency of at least about 1 picogram (pg) of protein expressed per milligram (mg) of total tissue protein per microgram (μg) of nucleic acid delivered. For example, the transfection efficiency of a nucleic acid: liposome complex can be at least about 10 pg of protein expressed per mg of total tissue protein per μg of nucleic acid delivered; or at least about 50 pg of protein expressed per mg of total tissue protein per μg of nucleic acid delivered. The transfection efficiency of the complex may be as low as 1 femtogram (fg) of protein expressed per mg of total tissue protein per μg of nucleic acid delivered, with the above amounts being more preferred.

[0102] In some embodiments, the liposomal delivery vehicle of the present invention is between about 100 and 500 nanometers (nm) in diameter. For example, the liposomal delivery vehicle can be between about 150 and 450 nm or between about 200 and 400 nm in diameter.

[0103] Suitable liposomes include any liposome, such as those commonly used in, for example, gene delivery methods known to those of skill in the art. In some embodiments, liposomal delivery vehicles comprise multilamellar vesicle (MLV) lipids, extruded lipids, or both. In some aspects, the liposomal delivery vehicle is cationic. Methods for preparation of MLVs are well known in the art. In some aspects, liposomal delivery vehicles comprise liposomes having a polycationic lipid composition (i.e., cationic liposomes) and/or liposomes having a cholesterol backbone conjugated to polyethylene glycol. Exemplary cationic liposome compositions include, but are not limited to, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)

and cholesterol, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol, 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)-imidazolinium chloride (DOTIM) and cholesterol, dimethyldioctadecylammonium bromide (DDAB) and cholesterol, and combinations thereof. In some aspects, the liposomal delivery vehicle comprises pairs of lipids selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and cholesterol; N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol; 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM) and cholesterol; and dimethyldioctadecylammonium bromide (DDAB) and cholesterol. In some aspects, the liposome composition for use as a delivery vehicle includes DOTIM and cholesterol.

[0104] Complexing a liposome with a herein described immunogenic nucleic acid plasmid may be achieved using methods standard in the art or as described in U.S. Patent No. 6,693,086, the contents of which are hereby incorporated by reference in their entirety. A suitable concentration of nucleic acid plasmid to add to a liposome includes a concentration effective for delivering a sufficient amount of the immunogenic nucleic acid plasmid into a subject such that a systemic immune response is elicited. For example, from about 0.1 μg to about 10 μg of immunogenic nucleic acid plasmid can be combined with about 8 nmol liposomes, from about 0.5 μg to about 5 μg of immunogenic nucleic acid plasmid can be combined with about 8 nmol liposomes, or about 1.0 μg of immunogenic nucleic acid plasmid can be combined with about 8 nmol liposomes. The ratio of immunogenic nucleic acid plasmid to lipid (μg immunogenic nucleic acid plasmid:nmol lipid) in a composition can be at least about 1:1 immunogenic nucleic acid plasmid:lipid by weight (e.g., 1 μg immunogenic nucleic acid plasmid:1 nmol lipid). For example, the ratio of immunogenic nucleic acid plasmid to lipids can be at least about 1:5, at least about 1:10, or at least about 1:20. Ratios expressed herein are based on the amount of lipid in the composition, and not on the total amount of lipid in the composition. The ratio of immunogenic nucleic acid plasmid to lipids in a composition of the invention is suitably from about 1:1 to about 1:80 immunogenic nucleic acid plasmid:lipid by weight; from about 1:2 to about 1:40 immunogenic nucleic acid plasmid:lipid by weight; from about 1:3 to about 1:30 immunogenic nucleic acid: lipid by weight; or from about 1:6 to about 1:15 immunogenic nucleic acid plasmid:lipid by weight.

[0105] The concentration of the immunomodulatory composition, if elevated above a threshold, can be cytotoxic. For this reason, the concentration of the immunomodulatory

composition as contemplated in the present disclosure is noncytotoxic, i.e., at a level below this threshold. "Cytotoxicity," as used herein, refers to an abnormal cellular state such as failure to thrive, retarded growth, irregular microscopic appearance, and/or decline in immunoresponsiveness. In some aspects, the concentration of the immunomodulatory composition is between about 0.1 and about 250 ng/ml. In some aspects the concentration is between about 0.1 and about 200 ng/ml. In some aspects, the concentration of the immunomodulatory composition is between about 0.1 and about 150 ng/ml. In other aspects, the concentration of the immunomodulatory composition is between about 0.1 and about 100 ng/ml. In still other aspects, the concentration of the immunomodulatory complex is between about 0.1 and about 50 ng/ml. In other aspects, the concentration of the immunomodulatory composition is between about 1 and about 250 ng/ml. In some aspects, the concentration of the immunomodulatory composition is between about 10 and about 250 ng/ml. In some aspects, the concentration of the immunomodulatory composition is between about 50 and about 250 ng/ml. In some aspects, the concentration of the immunomodulatory composition is between about 100 and about 250 ng/ml. In some aspects, the concentration of the immunomodulatory composition is between about 150 and about 250 ng/ml. In still other aspects, the concentration of the immunomodulatory composition is between about 200 and about 250 ng/ml. In some embodiments, the concentration of the immunomodulatory composition is about or less than 120 ng/ml. In some aspects, the concentration of the immunomodulatory composition is non-cytotoxic.

[0106] Further provided herein are pharmaceutical compositions comprising an immunostimulatory composition as described *supra* and a pharmaceutically acceptable carrier. The immunomodulatory composition may be administered before, simultaneously with, or after immunostimulatory oligonucleotide. The pharmaceutical carriers for the individual immunomodulatory composition and immunostimulatory oligonucleotide may be but need not be the same carrier. The pharmaceutically acceptable carrier adapts the composition for administration by a route selected from intravenous, intramuscular, intramammary, intradermal, intraperitoneal, subcutaneous, by spray, by aerosol, in ovo, mucosal, transdermal, by immersion, oral, intraocular, intratracheal, intranasal, pulmonary, rectal, or other means known to those skilled in the art. The pharmaceutically acceptable carrier(s) may be a diluent, adjuvant, excipient, or vehicle with which the immunostimulatory composition is, or immunomodulatory composition and immunostimulatory oligonucleotide are, administered. Such vehicles may be liquids, such as water and oils, including

those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. For example, 0.4% saline and 0.3% glycine can be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by conventional, well-known sterilization techniques (e.g., filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating, and coloring agents, etc. The concentration of the molecules of the invention in such pharmaceutical formulation may vary widely, i.e., from less than about 0.5%, usually to at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on required dose, fluid volumes, viscosities, etc., according to the particular mode of administration selected. Suitable vehicles and formulations, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in e.g. Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, (see especially pp. 958-989).

[0107] Methods are also provided herein for preparing the immunostimulatory composition, described *supra*, comprising combining the immunomodulator composition and the immunostimulatory oligonucleotide, to form the immunostimulatory composition; centrifuging the immunostimulatory composition to generate a supernatant and a pellet; and isolating the pellet.

[0108] Centrifuging the immunostimulatory composition will cause the sedimentation of the immunostimulatory composition. Isolating the pellet may be accomplished by pouring off the supernatant, pipetting off the supernatant, or removing the supernatant by other means so long as a portion of the pellet remains. It is to be expected that some pellet will be lost during the removal of the supernatant. Also, some immunostimulatory composition may remain in the supernatant even after centrifugation. In such a scenario, the supernatant may retain immunostimulatory properties. If immunostimulatory activity due to the presence of the immunostimulatory composition remains in the supernatant but it is desired to have nearly all of the immunostimulatory composition in the pellet, higher centrifugation speeds should be used. For example, if the supernatant contains immunostimulatory composition after centrifugation at 8,000 rpm, increasing the centrifugation to 14,000 rpm may bring down the remaining immunostimulatory composition.

[0109] Also provided herein are methods for stimulating toll-like receptor 21 (TLR21) comprising administering an immunostimulatory oligonucleotide and an immunomodulator

composition, wherein the immunostimulatory oligonucleotide comprises at least one CpG motif and an guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide, and wherein the immunomodulator composition comprises a noncoding nucleic acid plasmid and a cationic lipid delivery vehicle.

[0110] The immunostimulatory oligonucleotide and the immunomodulator composition can be administered by a route selected from intravenous, intramuscular, intramammary, intradermal, intraperitoneal, subcutaneous, by spray, by aerosol, in ovo, mucosal, transdermal, by immersion, oral, intraocular, intratracheal, intranasal, pulmonary, rectal, or other means known to those skilled in the art. In some aspects, the immunomodulator composition and the immunostimulatory oligonucleotide are present in synergistically effective amounts. The administration of the immunostimulatory composition and the immunomodulator composition may be sequential or simultaneous.

[0111] The concentration of the immunomodulator composition can be cytotoxic when above 250 μg/ml, and this cytotoxicity can more than offset any immunostimulatory effect of the immunomodulator. In some aspects of the present disclosures, the concentration of the immunomodulator is about 200 μg/ml. With administration of the immunostimulatory oligonucleotide, cytotoxic levels are not observed at or below the 10 μM range. Even higher concentrations of the immunostimulatory oligonucleotide may be tolerated by the recipient. In some aspects of the present disclosure the concentration of the immunostimulatory oligonucleotide is between about 10 μM and 0.5 μM. In some aspects the concentration of the immunostimulatory oligonucleotide is about 2 μM, and in some aspects, the concentration of the immunomodulator composition is greater than the concentration of the immunostimulatory oligonucleotide. Because cytotoxicity is a limiting factor with administration of the immunomodulator composition, in some aspects, the immunomodulator composition is present in non-cytotoxic amounts.

[0112] In each aspect of the methods presented herein, the immunomodulator composition and the immunostimulatory oligonucleotide can be any embodiment or aspect as described *supra*.

[0113] Also provided are methods for eliciting an immune response in a subject comprising administering any embodiment of the immunostimulatory composition described herein. Other embodiments included in the present disclosure include methods for eliciting an immune response in a subject comprising administering the immunostimulatory oligonucleotide and the immunomodulator composition described herein.

EXAMPLES

[0114] The following examples are provided to further describe some of the embodiments disclosed herein. The examples are intended to illustrate, not to limit, the disclosed embodiments.

[0115] The immunomodulator composition used in the following examples was a composition comprising a cationic lipid (DOTIM and cholesterol) and non-coding DNA (pMB75.6) (SEQ ID NO:266). The cationic lipid components were [1-[2-[9-(Z)-octadeceno-yloxy]]-2-[8](Z)-heptadecenyl]-3-[hydroxyethyl]imidazolinium chloride (DOTIM) and a synthetic neutral lipid cholesterol, formulated to produce liposomes approximately 200 nm in diameter (see, U.S. Pat. No. 6,693,086). The non-coding DNA component was a 4292 base-pair non-coding DNA plasmid (pMB75.6) (SEQ ID NO:266) produced in E. coli, which, being negatively charged, associates with the positively-charged (cationic) liposomes (see, U.S. Pat. No. 6,693,086). In the examples, the term "immunostimulatory nucleic acid plasmid" refers to pMB75.6.

Example 1: Combining TLR21-active oligodeoxynucleotides with an immunomodulator composition

[0116] The activity of the immunomodulator composition on TLR21 was explored. Specifically, HEK293-NFκB-bsd-cTLR21 cells were seeded into 384 well plates at 10,000 cells/well in 45 µl growth medium. These cells were exposed to the oligonucleotide dissolved in growth medium and incubated at 37° for 3-4 days. 10 µl of culture supernatant per well was transferred to a 384 well plate and 90 µl of 50 mM $NaHCO_3/Na_2CO_3$, 2 mM $MgCl_2$, 5mM *para*-nitrophenylphospate (pNP) pH 9.6 were added and reaction rates were determined by kinetic measurement of the temporal changes of the optical density at 405nM (mOD405nm/min).

[0117] The immunostimulatory nucleic acid plasmid alone proved to be inactive in the concentration range considered (2 µg/ml and lower), while liposomally formulated immunostimulatory nucleic acid plasmid (pDNA-F) showed a weak but clear signal with a bell-shaped curve, indicating its interaction with TLR21 (FIG. 2A). The TLR21-stimulatory activity was, however, several orders of magnitude lower compared to 5-Chol-GCGT3-TG4T (SEQ ID NO:1) (FIGs. 2A and 2B), an oligonucleotide ligand optimized for interacting with this receptor.

Table 2: ODN sequences

Immunostimulatory oligonucleotide	SEQ ID NO	Sequence
5Chol-GCGT3-TG4T (ODN1)	SEQ ID NO:1	XTGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT X = 5′-Cholesteryl
GCGT3-TG4T (ODN2)	SEQ ID NO:252	TGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT
2006-PTO (ODN3)	SEQ ID NO:3	tcgtcgttttgtcgttttgtcgtt

[0118] The activity of the immunomodulator composition pDNA-F on TLR21 suggests that this receptor may indeed be a component of the *in vivo* action of the immunomodulator composition, but because the immunomodulator composition is a rather weak ligand for TLR21, this receptor may not be the sole and dominant cognate receptor.

Example 2: Combination of 5-Chol-GCGT3-TG4T with the immunostimulatory nucleic acid plasmid and the immunomodulator composition

[0119] 200 µg/ml solutions of the immunostimulatory nucleic acid plasmid alone and the immunomodulator composition and 2 µM solutions of 5-Chol-GCGT3-TG4T were prepared and incubated for 2 h at 4°C. Subsequently, from this solution, serial 1:2 dilutions were prepared and administered to HEK293-bsd-cTLR21 cells starting at 20 nM plasmid concentration (and 2 µg/ml plasmid concentration) according to the protocol in Example 1 and compared to a sample containing only 5-Chol-GCGT3-TG4T. All samples showed strong TLR21 stimulatory activity, with the only sample showing slightly higher peak values and an EC_{50} of 2.44 pM (FIG. 3A, Table 3). Except for showing a slightly lower V_{max}, combination of 5-Chol-GCGT3-TG4T with the immunostimulatory nucleic acid plasmid, which by itself was totally inactive, led to little change in EC_{50}, compared to 5-Chol-GCGT3-TG4T alone (2.11 pM) (FIG. 3A, Table 3). By contrast, the liposome-containing sample (immunostimulatory oligonucleotide 5-Chol-GCGT3-TG4T and the immunomodulator composition) showed an activity maximum and a strong signal decrease at higher concentrations, (FIG. 3A). However, closer inspection of low concentrations (pM) revealed a defined activity plateau with a calculated EC_{50} of 1.04 pM (FIG. 3B, Table 3). In this concentration range, the immunomodulator composition is also totally inactive (FIG. 3B) and is, by itself, not responsible for the lower EC_{50}.

Table 3: Half-maximum effective concentration (EC_{50}) and maximum signal velocity (V_{max})

Immunostimulant	EC_{50} (pM)	Vmax milliOD 405nm/min (mOD405/min)
5-Chol-GCGT3-TG4T	2.44	338
5-Chol-GCGT3-TG4T-pDNA combination	2.11	260
5-Chol-GCGT3-TG4T-pDNA-F combination	1.04	254

[0120] The results suggest that the combination of the TLR21-stimulatory ODN 5-Chol-GCGT3-TG4T and non-cytotoxic concentrations of either immunostimulatory nucleic acid plasmid or the immunomodulator composition leads to active mixtures. Furthermore, the combination of the TLR21-stimulatory ODN 5-Chol-GCGT3-TG4T and the immunomodulator composition is synergistic with respect to the EC_{50} of TLR21 activation.

Example 3: Centrifugation of the immunomodulator composition and TLR21 activity

[0121] An immunomodulator composition solution of 200 µg/ml plasmid concentration was centrifuged for 2 hours at 4°C in an Eppendorf tabletop centrifuge at 14,000 rpm at 4°C. For comparison, a non-centrifuged aliquot was stored at 4°C for 2 hours. The supernatant was removed and stored, while the pellet was resuspended in an equivalent volume. Titrations starting at a 2 mg/ml plasmid content were prepared for use in the TLR21 assay as described in Example 1, as it had been established earlier that the immunomodulator composition possesses some weak TLR21 stimulatory activity (see Example 1).

[0122] Centrifugation of the immunomodulator composition resulted in a pellet that was difficult to resuspend with a pipette. All TLR21 stimulatory activity of immunomodulator composition was found in the pellet after centrifugation, and the supernatant was devoid of TLR21 activity (FIG. 4). The resuspended liposomes exhibited higher EC_{50}'s for TLR21 stimulation compared to the liposomes stored at 4°C. This effect may be due to changes in the liposomes after centrifugation (e.g., incomplete resuspension/dispersion).

Example 4: Combination of 5-Chol-GCGT3-TG4T with immunomodulator composition

[0123] An immunomodulator composition/5-Chol-GCGT3-TG4T solution having a 200 µg/ml plasmid concentration and 2 µM 5-Chol-GCGT3-TG4T was prepared. A 2 µM solution of 5-Chol-GCGT3-TG4T was also prepared. Both samples were incubated for 2 hours at 4°C. 100 µl

aliquots of these solutions were centrifuged in an Eppendorf tabletop centrifuge at 14,000 rpm at 4°C for 2 hours for use in Example 5, while the remainder of the incubations were stored at 4°C for analysis according to this Example 4. Both samples showed potent TLR21 stimulatory activity, but the immunomodulator composition/5-Chol-GCGT3-TG4T combination showed strongly decreasing signals at higher concentrations (FIG. 5A), likely a consequence of immunomodulator composition cytotoxicity. The respective V_{max} values were similar when the immunomodulator composition component of the sample was considered at low toxicity concentrations (FIG. 5B, Table 4). However, the calculated EC_{50} of the combination immunomodulator composition/5-Chol-GCGT3-TG4T was 4-fold lower than that of 5-Chol-GCGT3-TG4T alone (FIG. 5B, Table 4).

Table 4: Half-maximum effective concentration (EC_{50}) and maximum signal velocity (V_{max})

Immunostimulant	**EC_{50} (pM)**	**Vmax milliOD 405nm/min (mOD405/min)**
5-Chol-GCGT3-TG4T	3.2	158
5-Chol-GCGT3-TG4T (centrifugation supernatant)	2.5	160
5-Chol-GCGT3-TG4T (centrifugation pellet)	819	184
5-Chol-GCGT3-TG4T-pDNA-F combination	0.62	160
5-Chol-GCGT3-TG4T-pDNA-F combination (centrifugation supernatant)	5145	184
5-Chol-GCGT3-TG4T-pDNA-F combination (centrifugation pellet)	1.81	140

Example 5: Centrifugation of immunomodulator composition /5-Chol-GCGT3-TG4T and 5-Chol-GCGT3-TG4T

[0124] An immunomodulator composition/5-Chol-GCGT3-TG4T solution having a 200 µg/ml plasmid concentration and 2 µM 5-Chol-GCGT3-TG4T was prepared. A 2 µM solution of 5-Chol-GCGT3-TG4T was also prepared. Both samples were incubated for 2 hours at 4°C. 100 µl aliquots of these solutions were centrifuged in an Eppendorf tabletop centrifuge at 14,000 rpm at 4°C for 2 hours. The supernatants were removed and stored, while the pellets were resuspended in 100 µl. Subsequently, from these solutions, serial 1:2 dilutions were prepared and administered to HEK293-bsd-cTLR21 cells starting at 20 nM plasmid concentration (and 2 µg/ml plasmid concentration) and compared to a sample containing only 5-Chol-GCGT3-TG4T.

[0125] Centrifugation of the immunomodulator composition/5-Chol-GCGT3-TG4T combination led to a clearly visible pellet, while no visible pellet was observed for 5-Chol-GCGT3-TG4T. The immunomodulator composition/5-Chol-GCGT3-TG4T combination pellet ("pDNA-F 5Chol Pellet") was difficult to resuspend, but in the TLR21 assay as described in Example 1, it contained virtually all stimulating activity (FIGs. 6A and 6B), with only traces being detected in the supernatant ("pDNA-F 5Chol Uberstand") (FIG. 6B, Table 4), albeit with higher EC_{50} than the original sample ("pDNA-f F-Chol-GCGT3-TG4T") (Table 4). This result suggests that after mixing with immunomodulator composition, 5-Chol-GCGT3-TG4T is quantitatively physically associated with the liposomal fraction. 5-Chol-GCGT3-TG4T alone, which, when centrifuged, remains almost exclusively (an estimated 99%, Table 4) in the supernatant, as expected from a soluble compound (FIGs. 7A and 7B).

Example 6: Combination of 5-Chol-GCGT3-TG4T and immunomodulator composition

[0126] An immunostimulatory composition with 200 µg/ml plasmid concentration and 2 µM 5-Chol-GCGT3-TG4T was prepared. A 2 µM solution of 5-Chol-GCGT3-TG4T was also prepared. Both samples were incubated for 2 hours at 4°C. 100 µl aliquots of these solutions were centrifuged in an Eppendorf tabletop centrifuge at 14,000 rpm at 4°C for 2 hours for use in Example 7, while the remainder of the incubations were stored at 4°C for analysis according to this Example 6. The supernatants were removed and stored, while the pellets were resuspended in 200 µl. Subsequently, from these solutions, serial 1:2 dilutions were performed and administered to HEK293-bsd-cTLR21 cells for TLR21 analysis according to the protocol in Example 1. The starting plasmid concentration was 20 nM (and 2 µg/ml plasmid concentration) and compared to a sample containing only 5-Chol-GCGT3-TG4T.

[0127] Both samples ("5-Chol-GCGT3-TG4T" and "pDNA-F/5-Chol-GCGT3-TG4T") showed potent TLR21 stimulatory activity, but the immunomodulator composition/5-Chol-GCGT3-TG4T combination ("pDNA-F/5-Chol-GCGT3-TG4T") showed strongly decreasing signals at higher concentrations (FIG. 8A), likely a consequence of immunomodulator composition cytotoxicity. The respective V_{max} values were very similar when the stimulatory activity of immunomodulator composition-containing sample was considered at low toxicity concentrations (FIG. 8B, Table 5). However, the calculated EC_{50} of the combination immunomodulator

composition/5-Chol-GCGT3-TG4T was 2-fold lower than that of 5-Chol-GCGT3-TG4T ("5-Chol-GCGT3-TG4T") alone (FIG. 8B, Table 5).

Table 5: Half-maximum effective concentration (EC_{50}) and maximum signal velocity (V_{max})

Immunostimulant	EC_{50} picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
5-Chol-GCGT3-TG4T	2.2	120
5-Chol-GCGT3-TG4T (centrifugation supernatant)	2.7	153
5-Chol-GCGT3-TG4T (centrifugation pellet)	530	139
5-Chol-GCGT3-TG4T-pDNA-F combination	0.94	113
5-Chol-GCGT3-TG4T-pDNA-F combination (centrifugation supernatant)	14983	224
5-Chol-GCGT3-TG4T-pDNA-F combination (centrifugation pellet)	8.96	116

Example 7: Centrifugation of immunomodulator composition /5-Chol-GCGT3-TG4T and 5-Chol-GCGT3-TG4T

[0128] Centrifugation of the immunomodulator composition/5-Chol-GCGT3-TG4T combination led to a clearly visible pellet, while no visible pellet was observed for 5-Chol-GCGT3-TG4T. The immunomodulator composition/5-Chol-GCGT3-TG4T combination pellet was difficult to resuspend, but in a TLR21 assay as described in Example 1, it ("pDNA-F/5-Chol-GCGT3-TG4T pellet") contained virtually all of the stimulating activity (FIG. 9B), albeit with higher EC_{50} than the original sample (Table 5)), with only traces being detected in the supernatant ("pDNA-F/5-Chol-GCGT3-TG4T supernatant") (FIG. 9B, Table 5). This result suggests that after mixing with immunomodulator composition, the 5-Chol-GCGT3-TG4T is physically associated with the liposomal fraction. Both fractions were compared to non-centrifuged immunomodulator composition/5-Chol-GCGT3-TG4T ("pDNA-F/5-Chol-GCGT3-TG4T")

Example 8: Combination of 5-Chol-GCGT3-TG4T with immunomodulator composition

[0129] An immunomodulator composition solution of 200 µg/ml plasmid concentration and 2 µM 5-Chol-GCGT3-TG4T was prepared. A 2 µM 5-Chol-GCGT3-TG4T sample also was prepared. Both samples were incubated for 2 hours at 4°C. 100 µl aliquots of these solutions were centrifuged in an Eppendorf tabletop centrifuge at 14,000 rpm at 4°C for 2 hours for use in Example 9, while the remainder of the incubations were stored at 4°C for analysis according to this Example 8. The supernatants were removed and stored, while the pellets were resuspended in 100 µl. Subsequently, from these solutions, serial 1:2 dilutions were prepared and administered to HEK293-bsd-cTLR21 cells according to the protocol of Example 1, starting at 20 nM plasmid concentration (and 2 µg/ml plasmid concentration) and compared to a sample containing only 5-Chol-GCGT3-TG4T.

[0130] Both samples showed potent TLR21 stimulatory activity, but the 5-Chol-GCGT3-TG4T/immunomodulator composition combination ("pDNA-F/5-Chol-GCGT3-TG4T") showed strongly decreasing signals at higher concentrations (FIG. 10A), similar to that of the immunomodulator by itself ("pDNA-F") and likely a consequence of immunomodulator composition cytotoxicity. The immunostimulatory oligonucleotide ("5-Chol-GCGT3-TG4T") exhibited greater stimulatory activity at higher concentrations than did either sample containing the immunomodulator composition. The respective V_{max} values were very similar when the stimulatory activity of immunomodulator composition component of the sample was considered at low toxicity concentrations (FIG. 10B, Table 6). However, the calculated EC_{50} of the combination immunomodulator composition/5-Chol-GCGT3-TG4T ("pDNA-F/5-Chol-GCGT3-TG4T") was 4-fold lower than that of 5-Chol-GCGT3-TG4T alone ("5-Chol-GCGT3-TG4T") (FIG. 10B, Table 6). The immunomodulator composition alone ("Bay 98-F") showed only minimal activity whose additive effect could not explain the increased activity of immunomodulator composition/5-Chol-GCGT3-TG4T *versus* 5-Chol-GCGT3-TG4T alone.

Table 6: Half-maximum effective concentration (EC_{50}) and maximum signal velocity (V_{max}):

Immunostimulant	**EC_{50} picomolar (pM)**	**Vmax milliOD 405nm/min (mOD405/min)**
5-Chol-GCGT3-TG4T	2.47	53

5-Chol-GCGT3-TG4T-pDNA-F combination	0.59	51
5-Chol-GCGT3-TG4T-pDNA-F combination (centrifugation supernatant)	22.0	47
5-Chol-GCGT3-TG4T-pDNA-F combination (centrifugation pellet)	1.06	47

Example 9: Centrifugation of immunomodulator composition

[0131] Centrifugation of immunomodulator composition/5-Chol-GCGT3-TG4T combination led to a clearly visible pellet, while for 5-Chol-GCGT3-TG4T, no visible pellet was observed. The immunomodulator composition/5-Chol-GCGT3-TG4T combination pellet ("pDNA-F/5-Chol-GCGT3-TG4T Pellet") was difficult to resuspend, but in a TLR21 assay as described in Example 1, it contained > 95% of the stimulating activity (FIG. 11B), albeit with higher EC_{50} than the original sample, with only a small fraction (< 5%) being detected in the supernatant ("pDNA-F/5-Chol-GCGT3-TG4T Supernatant") (FIG. 11B, Table 6) and only slightly less than the non-centrifuged sample at low immunomodulator composition concentrations ("pDNA-F/5-Chol-GCGT3-TG4T"). This result suggests that after mixing with immunomodulator composition, 5-Chol-GCGT3-TG4T is quantitatively physically associated with the liposomal fraction.

Example 10: Combination of GCGT3-TG4T with immunomodulator composition

[0132] An immunomodulator composition solution of 200 μg/ml plasmid concentration and 2 μM GCGT3-TG4T (SEQ ID NO:252; the same oligonucleotide sequence as 5-Chol- GCGT3-TG4T (SEQ ID NO:1) but without the cholesteryl modification) was prepared. Also, a 2μM GCGT3-TG4T sample was prepared, and both samples were incubated for 2 hours at 4°C. 100 μl aliquots of these solutions were centrifuged in an Eppendorf tabletop centrifuge at 14,000 rpm at 4°C for 2 hours for use in Example 11, while the remainder of the incubations were stored at 4°C for analysis according to this Example 10. The supernatants were removed and stored, while the pellets were resuspended in 100 μl. Subsequently, from these solutions, serial 1:2 dilutions were prepared and administered to HEK293-bsd-cTLR21 cells according to the protocol in Example 1, starting at 20 nM plasmid concentration (and 2 μg/ml plasmid concentration) and compared to a sample containing only GCGT3-TG4T.

[0133] Both samples showed potent TLR21 stimulatory activity, but the GCGT3-TG4T/immunomodulator composition combination ("pDNA-F/5-Chol-GCGT3-TG4T") showed strongly decreasing signals at higher concentrations (FIG. 12A), likely a consequence of immunomodulator composition cytotoxicity. The respective V_{max} values were very similar, when the stimulatory activity of immunomodulator composition component of the sample was considered at low toxicity concentrations (FIG. 12B, Table 7). However, the calculated EC_{50} of the combination immunomodulator composition/GCGT3-TG4T was more than 4-fold lower than that of GCGT3-TG4T alone (Table 7). The immunomodulator composition alone ("pDNA-F") showed only minimal activity whose additive effect could not explain the increased activity of immunomodulator composition/GCGT3-TG4T *versus* GCGT3-TG4T alone.

Table 7: Half-maximum effective concentration (EC_{50}) and maximum signal velocity (V_{max})

Immunostimulant	**EC_{50} picomolar (pM)**	**Vmax milliOD 405nm/min (mOD405/min)**
GCGT3-TG4T	324	56
GCGT3-TG4T-pDNA-F combination	69.4	51
GCGT3-TG4T-pDNA-F combination (centrifugation supernatant)	860	43
GCGT3-TG4T-pDNA-F combination (centrifugation pellet)	252	36

Example 11: Centrifugation of immunomodulator composition/GCGT3-TG4T

[0134] Centrifugation of the immunomodulator composition/GCGT3-TG4T combination led to a clearly visible pellet. The immunomodulator composition/GCGT3-TG4T combination pellet was difficult to resuspend, but in a TLR21 assay as described in Example 1 it ("pDNA-F/GCGT3-TG4T Pellet") contained > 3 x more stimulating activity (FIG. 13B), albeit with higher EC_{50} than the original sample ("pDNA-F/GCGT3-TG4T") than the supernatant ("pDNA-F/GCGT3-TG4T Supernatant") (FIG. 13B, Table 7). This result suggests that after mixing with immunomodulator composition, the GCGT3-TG4T is quantitatively physically associated with the liposomal fraction, although perhaps not as efficiently as the cholesteryl-derivatized 5-Chol-GCGT3-TG4T.

[0135] Those skilled in the art will appreciate that numerous changes and modifications can be made to the preferred embodiments of the invention and that such changes and modifications

can be made without departing from the spirit of the invention. It is, therefore, intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

[0136] The disclosures of each patent, patent application, and publication cited or described in this document are hereby incorporated herein by reference, in its entirety.

Example 12: *In vivo* study of efficacy of immune stimulants in a Newcastle disease vaccination model in chickens

[0137] To determine the suitability and efficacy of ODN1, ODN2, and ODN3 as immune stimulants, each was tested in three different concentrations.

[0138] The following immune stimulants were investigated:

ODN1:[CholTEG]-TGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT ("5Chol-GCGT3-TG4T") (SEQ ID NO:1) ([CholTEG]=5'-triethyleneglycol-linked cholesteryl modification),

ODN2:TGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT ("GCGT3-TG4T") (SEQ ID NO:252),

ODN3:tcgtcgttttgtcgttttgtcgtt ("2006-PTO") (SEQ ID NO:3).

[0139] Each immune stimulant was added to an oil emulsion containing a suboptimal concentration of an inactivated Newcastle disease virus (NDV) according to **Table 9**. For the preparaton of the suboptimal NDV vaccine, the NDV antigen batch was diluted 50 times in NDV-negative allantoic fluid (AF). The efficacies of ODN1, ODN2, and ODN3 in combination with a suboptimal dosage of a Newcastle disease vaccine were tested in SPF layer chickens (Leghorn). The serological response was measured and compared to the similar suboptimal NDV vaccine without the immune stimulant. The antibody titre was determined at different time points after vaccination to investigate whether the addition of the immune stimulants leads to an earlier immune response. To determine the most optimal dosage of the three ODNs, each was supplemented in three different doses of 100 ng, 1000 ng and 5000 ng to the suboptimal NDV vaccine, resulting in nine immune stimulant groups. Besides these nine immune stimulant groups, five control groups were incorporated in this study, consisting of a suboptimal NDV vaccine without immune stimulant group, the non-diluted NDV vaccine group, a negative control group (immune stimulants in

combination with adjuvant) and two positive control groups with polyinosinic:polycytidylic acid (poly I:C) at two different concentrations (**Table 8**).

[0001] The following parameters were tested: health of the chickens (data not shown) and serology by the Haemagglutination inhibition (HI) assay.

Table 8: Study Design

Test Article / *Control Item*	Test Group	Number (n)
Suboptimal NDV+ ODN1 100ng	T01	10
Suboptimal NDV+ ODN1 1000 ng	T02	10
Suboptimal NDV+ ODN1 5000 ng	T03	10
Suboptimal NDV+ ODN2 100 ng	T04	10
Suboptimal NDV+ ODN2 1000 ng	T05	10
Suboptimal NDV+ ODN2 5000 ng	T06	10
Suboptimal NDV+ ODN3 100 ng	T07	10
Suboptimal NDV+ ODN3 1000 ng	T08	10
Suboptimal NDV+ ODN3 5000 ng	T09	10
Suboptimal NDV	T10	10
Optimal NDV (non-diluted vaccine)	T11	10
*ODN1 5000 ng + Adjuvant**	T12a	3
*ODN2 5000 ng + Adjuvant**	T12b	3
*ODN3 5000 ng + Adjuvant**	T12c	3
*Adjuvant alone (Stimune)**	T12d	1
Suboptimal NDV+ 10 µg Poly I:C	T13	9
Suboptimal NDV+ 100 µg Poly I:C	T14	9

*3 animals were allocated as control for each immune stimulant in combination with the adjuvant (Stimune). One animal received the adjuvant only.
All animals arrived at 3 weeks old.
All animals were vaccinated at 5 weeks old. All vaccinations were performed at day 0 by intramuscular injection.
Blood sampling/serology was performed on days 0 (before vaccination), 7, 14, and 21.
Clinical scoring of all animals was performed daily.

[0140] Chickens enrolled in treatment groups T01-T14 received the Test Article or Control Item according to the study design. In groups T13 and T14, nine instead of ten chickens per group were vaccinated due to the loss of two animals before the start of the study.

[0141] Chickens allocated to treatment groups T01, T02, T03, T04, T05, T06, T07, T08 and T09 were vaccinated with a suboptimal NDV suspension containing 1 of 3 different immune stimulants (ODNs), each in 3 different concentrations (100, 1000, 5000 ng/dose). For the preparation of the water in oil emulsions, the NDV antigen suspension and immune stimulant (water phase) were formulated together with the adjuvant Stimune (oil phase) at a ratio of 4:5 (**Table 9**).

Table 9: Preparation of Test Articles and Control Items

		Water Phase				Oil Phase		
Group	Name	NDV batch (µl)	Neg. AF (µl)	Stimune 600 ng/µl (µl)	Total volume water phase (ml)	Add volume water phase to Stimune (ml)	Stimune (ml)	Total (ml)
T01	ODN1 100 ng	100	4896	4	5	4	5	9
T02	ODN1 1000 ng	100	4862	38	5	4	5	9
T03	ODN1 5000 ng	100	4712	188	5	4	5	9
T04	ODN2 100 ng	100	4896	4	5	4	5	9
T05	ODN2 1000 ng	100	4862	38	5	4	5	9
T06	ODN2 5000 ng	100	4712	188	5	4	5	9
T07	ODN3 100 ng	100	4896	4	5	4	5	9
T08	ODN3 1000 ng	100	4862	38	5	4	5	9
T09	ODN3 5000 ng	100	4712	188	5	4	5	9
T10	Suboptimal vaccine	100	4900	0	5	4	5	9
T11	Non diluted vaccine	5000	0	0	5	4	5	9
T12a	ODN1 5000 ng in Stimune	-	2887	113	3	2	2.5	4.5
T12b	ODN2 5000	-	2887	113	3	2	2.5	4.5

	ng in Stimune							
T12c	ODN3 5000 ng in Stimune	-	2887	113	3	2	2.5	4.5
T12d	Dilution buffer (PBS) in Stimune	-	2887	113	3	0.8	1	1.8
T13	PolyI:C 10 µg	100	4877	23	5	4	5	9
T14	PolyI:C 100 µg	100	4675	225	5	4	5	9

ODN Preparation to 600 ng/µl				
		100 µM ODN (µl)	Dilution Buffer (µl)	Volume Stock 600 ng/µl (µl)
ODN1	GCGT3-TG4T-5Chol (SEQ ID NO:1, see Table 1)	204	196	400
ODN2	GCGT3-TG4T (SEQ ID NO:252, see Table 1)	216	184	400
ODN3	2006-PTO (SEQ ID NO: 3, see Table 1)	312	88	400
Poly I:C 10 µg/µl				
		Lyophilized Powder (mg)	Physiological Salt Solution (ml)	Volume Stock 10 µg/µl (µl)
Control	Poly I:C (P0913)	10	1	1000
Lot #s:	116M4118V		#16TK5011	10 min 50 , 60 min RT (re-annealing)
				storage at -20

[0142] Chickens allocated to control group of T10 were vaccinated with a suboptimal NDV suspension without immune stimulant in adjuvant (Stimune) at a ratio of 4:5.

[0143] Chickens allocated to control group of T11 were vaccinated with a non-diluted NDV suspension without immune stimulant in adjuvant (Stimune) at a ratio of 4:5.

[0144] Chickens allocated to group T12 were vaccinated with immune stimulant 1 (3 chickens), immune stimulant 2 (3 chickens) and immune stimulant 3 (3 chickens) in adjuvant (Stimune) at a ratio of 4:5. One chicken was vaccinated with dilution buffer (proprietary) in adjuvant (Stimune).

[0145] Chickens allocated to control groups of T13 (n=9) and T14 (n=9) were vaccinated with a suboptimal NDV suspension of NDV in combination with Poly I:C in two concentrations (10,000 ng and 100 µg) in adjuvant (Stimune) at a ratio of 4:5.

Test Article or Control Item Administration

[0146] The inactivated NDV strain Ulster suspension stored at -70°C was thawed and diluted 50 times in negative allantoic fluid to create the suboptimal vaccine dose. Immune stimulants were added according to the study design. The resulting water phases were mixed with Stimune in a ratio of 4:5 according to the vaccination preparation scheme shown in **Table 9**. During preparation, all vaccine ingredients with the exception of the Stimune adjuvant were placed in melting ice. The formulated vaccines were injected (0.5 ml, intramuscular) directly after preparation.

[0147] General health was monitored by an experienced bio-technician daily from day of arrival until the end of the study.

Serum blood sampling

[0148] Blood samples for serology were collected from all chickens on study days 0 (prior to vaccination), 7, 14 and 21. Blood samples were labelled with the study number, a unique sample identification and the date of collection. Depending on the amount of the drawn blood volume, sera were aliquoted in two aliquots of approximately 0.5 ml and stored at -20 ±5 .

Haemagglutination inhibition (HI) assay

[0149] In brief, dilution series of sera were incubated with 8 HAU (haemagglutinating units) of NDV strain Ulster at room temperature for 60 minutes. The HAU were titrated before each assay. Thereafter, chicken erythrocytes were added and agglutination was scored after incubation at 4°C for 45 minutes. A negative control serum and three positive control sera, with low, intermediate and high antibody titres were included in each assay.

[0150] The HI titre results were expressed as the reciprocal of the highest serum dilution completely inhibiting agglutination, which were logarithmically transformed to the final Log2 titres.

Statistics

[0151] Logarithmically transformed HI results were summarized per animal (see **Tables 62-65**). Per treatment group, the mean and standard deviation of the antibody titres were calculated. The statistical analysis was performed with the non-parametric Mann-Whitney t-test.

Results

[0152] No clinical symptoms or adverse events related to the vaccination were observed in all groups, all chickens appeared healthy during the entire study period.

[0153] Two chickens, however were scored with minor injuries due to pecking behaviour, which started 6 days before the start of the study. On the day of vaccination these chickens were allocated to the Poly I:C groups T13 (#11658) and T14 (#11676). Recovery took place within one week after vaccination.

ODN1, GCGT3-TG4T-5Chol

[0154] The individual HI results expressed as Log2 titres of the 100 ng, 1000 ng and 5000 ng ODN1 dose groups are indicated in **Table 10**. The mean HI titres and standard deviation of these groups are indicated in **FIG. 14** (days 14 and 21 post vaccination (pv)) and **FIG. 15** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0155] The GCGT3-TG4T-5Chol groups showed significantly higher HI titres compared to the diluted NDV vaccine (mean HI titre: 4.8 Log2/SD 1.0). At day 14 pv this was the case for all three doses; 100 ng: mean HI titre 6.2 Log2/SD 1.4 ($p=0.0214$), 1000 ng: mean HI titre 6.9 Log2/SD 1.1 ($p=0.0003$) and 5000 ng: mean HI 5.9 Log2/SD 0.7 ($p=0.0243$).

[0156] At day 21 pv, however, no significant differences were observed for all concentrations; 100 ng: mean HI titre 6.9 Log2 /SD 0.8 ($p=0.1995$); 1000 ng: mean HI titre 7.3 Log2/SD 0.9 ($p=0.0527$); and 5000 ng: mean HI 6.7 Log2/SD 0.9 ($p=0.4523$) when comparing to the NDV vaccine; HI titre 6.2 Log2/ SD1.0. (**FIG. 14**), although the 1000 ng concentration is very close to significance.

Table 10:

Results	duplo HI		HI 1	HI2		HI 1	HI2		HI 1	HI2	HI3		HI 1	HI2	HI3	
group	Treatment	animal	d0	d0	mean	d7	d7	mean	d14	d14	d14	mean	d21	d21	d21	mean
T01	GCGT3-TG4T-5Chol 100 ng	11402	0	0	**0**	1	1	**1**	7	7	7	**7.0**	7	7	7	**7.0**
		11404	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	7	**7.0**
		11406	0	0	**0**	0	0	**0**	3	4	4	**3.7**	6	6	6	**6.0**
		11408	0	0	**0**	0	0	**0**	7	8	8	**7.7**	8	9	7	**8.0**
		11410	0	0	**0**	0	0	**0**	5	6	6	**5.7**	7	7	7	**7.0**
		11412	0	0	**0**	0	0	**0**	6	6	8	**6.7**	6	6	6	**6.0**
		11414	0	0	**0**	0	0	**0**	5	5	6	**5.3**	7	7	6	**6.7**
		11416	0	0	**0**	0	0	**0**	8	7	8	**7.7**	8	8	8	**8.0**
		11418	0	0	**0**	0	0	**0**	5	4	4	**4.3**	6	6	6	**6.0**
		11420	0	0	**0**	0	0	**0**	8	7	7	**7.3**	7	7	8	**7.3**
		mean			**0.0**			**0.1**				**6.2**				**6.9**
		SD			**0.0**			**0.3**				**1.4**				**0.8**
T02	GCGT3-TG4T-5Chol 1000 ng	11422	0	0	**0**	0	0	**0**	7	6	7	**6.7**	6	6	6	**6.0**
		11424	0	0	**0**	0	0	**0**	8	7	7	**7.3**	9	7	8	**8.0**
		11426	0	0	**0**	0	0	**0**	6	5	5	**5.3**	6	6	6	**6.0**
		11428	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	7	**7.0**
		11430	0	0	**0**	1	1	**1**	10	9	10	**9.7**	8	8	9	**8.3**
		11432	0	0	**0**	0	0	**0**	7	6	7	**6.7**	7	7	7	**7.0**
		11434	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	7	7	**7.0**
		11436	0	0	**0**	0	0	**0**	7	6	7	**6.7**	8	7	9	**8.0**
		11438	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	7	7	**7.0**
		11440	0	0	**0**	0	0	**0**	7	7	7	**7.0**	8	8	9	**8.3**
		mean			**0.0**			**0.1**				**6.9**				**7.3**
		SD			**0.0**			**0.3**				**1.1**				**0.9**
T03	GCGT3-TG4T-5Chol 5000 ng	11442	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	8	**7.3**
		11444	0	0	**0**	0	0	**0**	5	5	5	**5.0**	6	6	6	**6.0**
		11446	0	0	**0**	0	0	**0**	5	4	5	**4.7**	5	5	6	**5.3**
		11448	0	0	**0**	0	0	**0**	7	7	7	**7.0**	8	8	9	**8.3**
		11450	0	0	**0**	0	0	**0**	6	5	5	**5.3**	6	6	7	**6.3**
		11452	0	0	**0**	0	0	**0**	6	5	6	**5.7**	7	7	7	**7.0**
		11454	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	6	7	**6.7**
		11456	0	0	**0**	0	0	**0**	6	6	6	**6.0**	6	6	6	**6.0**
		11458	0	0	**0**	0	0	**0**	6	5	6	**5.7**	6	6	7	**6.3**
		11460	0	0	**0**	0	0	**0**	7	6	7	**6.7**	7	7	8	**7.3**
		mean			**0.0**			**0.0**				**5.9**				**6.7**
		SD			**0.0**			**0.0**				**0.7**				**0.9**

ODN2, GCGT3-TG4T

[0157] The individual HI results expressed as Log2 titres of the 100 ng, 1000 ng and 5000 ng ODN1 dose groups are indicated in **Table 11**. The mean HI titres and standard deviation of these groups are indicated in **FIG. 16** (days 14 and 21 pv) and **FIG. 17** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0158] The ODN2, GCGT3-TG4T groups showed significantly higher HI titres compared to the diluted NDV vaccine (mean HI titre: 4.8 Log_2/SD 1.0). This was the case at day 14 post vaccination for all three doses; 100 ng: mean HI titre 7.1 Log_2/SD 1.2 (p=0.0003), 1000 ng: mean HI titre 6.4 Log_2/SD 0.7 (p=0.0027) and 5000 ng: mean HI titre 6.1 Log_2/SD 1.1 (p=0.0236). At day 21 significant differences were only observed at the 100 ng dose with a mean HI titre of 7.6 Log_2/SD 0.8 (p=0.0083) when compared to the NDV vaccine (HI titre 6.2 Log_2/SD 1.0). The mean HI titres

for the 1000 ng and 5000 ng were 7.1 Log_2/0.6 (p=0.0696) and 7.2 Log_2/SD 1.0 (p=0.0956) respectively (**FIG. 16**).

Table 11:

T04	GCGT3-TG4T 100 ng	11462	0	0	**0**	0	0	**0**	7	6	7	**6.7**	7	7	8	**7.3**
		11464	0	0	**0**	0	0	**0**	8	7	8	**7.7**	7	8	8	**7.7**
		11466	0	0	**0**	0	0	**0**	7	6	6	**6.3**	8	8	7	**7.7**
		11468	0	0	**0**	0	0	**0**	8	7	8	**7.7**	8	9	8	**8.3**
		11470	0	0	**0**	0	0	**0**	7	6	7	**6.7**	7	7	7	**7.0**
		11472	0	0	**0**	0	0	**0**	10	10	9	**9.7**	10	9	8	**9.0**
		11474	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	7	7	**7.0**
		11476	0	0	**0**	0	0	**0**	6	5	5	**5.3**	7	7	6	**6.7**
		11478	0	0	**0**	0	0	**0**	8	6	6	**6.7**	7	7	7	**7.0**
		11480	0	0	**0**	0	0	**0**	9	8	7	**8.0**	9	9	8	**8.7**
		mean			**0.0**			**0.0**				**7.1**				**7.6**
		SD			**0.0**			**0.0**				**1.2**				**0.8**
T05	GCGT3-TG4T 1000 ng	11482	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	7	7	**7.0**
		11484	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	7	**7.0**
		11486	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	7	7	**7.0**
		11488	0	0	**0**	0	0	**0**	6	8	6	**6.7**	8	8	8	**8.0**
		11490	0	0	**0**	0	0	**0**	5	5	5	**5.0**	6	6	6	**6.0**
		11492	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	8	**7.3**
		11494	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	7	**7.0**
		11496	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	8	7	**7.3**
		11498	0	0	**0**	0	0	**0**	8	7	7	**7.3**	9	7	8	**8.0**
		11500	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	6	7	**6.7**
		mean			**0.0**			**0.0**				**6.4**				**7.1**
		SD			**0.0**			**0.0**				**0.7**				**0.6**
T06	GCGT3-TG4T 5000 ng	11502	0	0	**0**	0	0	**0**	8	7	7	**7.3**	10	8	9	**9.0**
		11504	0	0	**0**	0	0	**0**	7	7	6	**6.7**	8	7	7	**7.3**
		11506	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	6	7	**6.7**
		11508	0	0	**0**	0	0	**0**	6	5	5	**5.3**	8	6	7	**7.0**
		11510	0	0	**0**	0	0	**0**	8	7	7	**7.3**	9	8	8	**8.3**
		11512	0	0	**0**	0	0	**0**	8	6	7	**7.0**	9	7	8	**8.0**
		11514	0	0	**0**	0	0	**0**	5	5	5	**5.0**	6	6	7	**6.3**
		11516	0	0	**0**	0	0	**0**	7	6	6	**6.3**	7	7	7	**7.0**
		11518	0	0	**0**	0	0	**0**	6	5	5	**5.3**	7	6	8	**7.0**
		11520	0	0	**0**	0	0	**0**	4	4	4	**4.0**	6	5	6	**5.7**
		mean			**0.0**			**0.0**				**6.1**				**7.2**
		SD			**0.0**			**0.0**				**1.1**				**1.0**

ODN3, 2006-PTO

[0159] The individual HI results expressed as Log2 titres of the 100 ng, 1000 ng and 5000 ng ODN1 dose groups measured are indicated in **Table 12**. During the triplicate HI assay performance an outlier result was observed for animal 11570 on day 21, this was most likely caused by a pipetting error (not enough AF added) and therefore this result was omitted from the final analysis (highlighted in **Table 12**). Thus, for this animal and date the mean HI titre was based on the duplicate measurement.

[0160] The mean HI titres and standard deviation of these groups are indicated in **FIG. 18** (days 14 and 21 pv) and **FIG. 19** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0161] The ODN3, 2006-PTO groups showed significantly higher HI titres compared to the diluted NDV vaccine (mean HI titre: 4.8 Log2/SD 1.0). This was the case at day 14 post vaccination for two doses; 1000 ng: mean HI titre: 6.3 Log2/SD 1.2 (p=0.0081) and 5000 ng: mean HI titre: 6.2 Log2/SD 0.8 (p=0.0059).The mean HI titre of the 100 ng dose was 5.3 Log2/SD 0.5 (p=0.2090). At day 21 pv significant differences were only measured at the 5000 ng: mean HI titre 7.3 Log2/SD 0.6 (p=0.0296). No significant differences were observed at the 100 ng and 1000 ng doses, with mean HI titres of 6.6 Log2/SD 0.5 (p=0.7183) and 6.8 Log2/SD 1.1 (p=0.1685) respectively, when comparing to the NDV vaccine; HI titre 6.2 Log2/SD 1.0 (**FIG. 18**).

Table 12:

T07	2006-PTO 100 ng	11522	0	0	**0**	0	0	**0**	6	5	5	**5.3**	6	6	6	**6.0**
		11524	0	0	**0**	0	0	**0**	5	5	6	**5.3**	6	6	6	**6.0**
		11526	0	0	**0**	0	0	**0**	6	5	6	**5.7**	7	7	7	**7.0**
		11528	0	0	**0**	0	0	**0**	5	5	5	**5.0**	6	7	6	**6.3**
		11530	0	0	**0**	0	0	**0**	6	5	7	**6.0**	7	7	7	**7.0**
		11532	0	0	**0**	0	0	**0**	5	5	5	**5.0**	5	6	6	**5.7**
		11534	0	0	**0**	0	0	**0**	5	5	6	**5.3**	7	7	7	**7.0**
		11536	0	0	**0**	0	0	**0**	5	5	6	**5.3**	7	7	7	**7.0**
		11538	0	0	**0**	0	0	**0**	4	4	5	**4.3**	7	6	7	**6.7**
		11540	0	0	**0**	0	0	**0**	6	5	6	**5.7**	7	7	7	**7.0**
		mean			**0.0**			**0.0**				**5.3**				**6.6**
		SD			**0.0**			**0.0**				**0.5**				**0.5**
T08	2006-PTO 1000 ng	11542	0	0	**0**	0	0	**0**	6	5	6	**5.7**	6	6	7	**6.3**
		11544	0	0	**0**	0	0	**0**	6	4	6	**5.3**	7	7	7	**7.0**
		11546	0	0	**0**	0	0	**0**	4	4	5	**4.3**	4	5	4	**4.3**
		11548	0	0	**0**	0	0	**0**	5	5	6	**5.3**	6	6	7	**6.3**
		11550	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	8	**7.3**
		11552	0	0	**0**	0	0	**0**	7	7	8	**7.3**	7	7	8	**7.3**
		11554	0	0	**0**	0	0	**0**	8	8	9	**8.3**	8	8	9	**8.3**
		11556	0	0	**0**	0	0	**0**	6	6	6	**6.0**	6	6	6	**6.0**
		11558	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	8	**7.3**
		11560	0	0	**0**	0	0	**0**	7	7	7	**7.0**	8	8	8	**8.0**
		mean			**0.0**			**0.0**				**6.3**				**6.8**
		SD			**0.0**			**0.0**				**1.2**				**1.1**
T09	2006-PTO 5000 ng	11562	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	7	8	**7.3**
		11564	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	8	**7.3**
		11566	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	7	**7.0**
		11568	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	7	**7.0**
		11570	0	0	**0**	0	0	**0**	5	5	6	**5.3**	7	11	7	**7.0**
		11572	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	8	8	**7.7**
		11574	0	0	**0**	0	0	**0**	5	5	6	**5.3**	6	7	7	**6.7**
		11576	0	0	**0**	0	0	**0**	8	8	9	**8.3**	8	10	9	**9.0**
		11578	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	7	**7.0**
		11580	0	0	**0**	1	1	**1**	5	5	7	**5.7**	7	7	8	**7.3**
		mean			**0.0**			**0.1**				**6.2**				**7.3**
		SD			**0.0**			**0.3**				**0.8**				**0.6**

Control groups

[0162] The individual HI results expressed as Log2 titres of the 10 µg and 100 µg Poly I:C dose groups, the diluted and non-diluted NDV vaccines and the negative control groups are indicated in **Table 13**. The mean HI titres and standard deviation of these groups are indicated in

FIG. 20 (days 14 and 21 pv) and **FIG. 21** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0163] For Poly I:C, the positive control groups, significantly higher HI titres were only observed at the 100 µg dose: HI titre 7.5 Log2/SD 0.4 at day 21 (p=0.0053) when compared with the NDV vaccine (6.2 Log2/SD 1.0). The mean HI titres at day 14 pv of the 10 µg and 100 µg dose groups were 5.8 Log2/SD 1.3 (p=0.1859) and 5.5 Log2/SD 0.8 (p=0.1609) respectively. The mean HI titre of the 10 µg dose group at day 21 pv was 6.4 Log2/SD 1.3 (p=0.7273). Significant differences ($p < 0.0001$) were observed between the non-diluted NDV vaccine (8.3/SD 0.5 and 8.5 Log2/SD 0.7) and the negative control group compared to the diluted NDV group at days 14 and 21 post vaccination (4.8/SD 1.0 and 6.2 Log2/ SD 1.0, respectively) (**FIG. 20**).

Table 13:

T10	Suboptimal vaccine (1:50)	11582	0	0	**0**	0	0	**0**	4	4	4	**4.0**	6	5	6	**5.7**
		11584	0	0	**0**	0	0	**0**	5	6	5	**5.3**	7	7	7	**7.0**
		11586	0	0	**0**	0	0	**0**	5	5	6	**5.3**	5	5	6	**5.3**
		11588	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	6	8	**7.0**
		11590	0	0	**0**	0	0	**0**	4	4	5	**4.3**	6	6	6	**6.0**
		11592	0	0	**0**	0	0	**0**	5	5	5	**5.0**	7	7	8	**7.3**
		11594	0	0	**0**	0	0	**0**	4	4	5	**4.3**	6	7	8	**7.0**
		11596	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	8	**7.3**
		11598	0	0	**0**	0	0	**0**	4	4	4	**4.0**	4	4	5	**4.3**
		11600	0	0	**0**	0	0	**0**	3	3	4	**3.3**	5	5	6	**5.3**
		mean			**0.0**			**0.0**				**4.8**				**6.2**
		SD			**0.0**			**0.0**				**1.0**				**1.0**
T11	Non diluted vaccine	11602	0	0	**0**	0	0	**0**	8	8	8	**8.0**	9	9	10	**9.3**
		11604	0	0	**0**	0	0	**0**	9	9	8	**8.7**	8	9	10	**9.0**
		11606	0	0	**0**	0	0	**0**	7	7	8	**7.3**	8	8	9	**8.3**
		11608	0	0	**0**	0	0	**0**	8	9	9	**8.7**	9	9	10	**9.3**
		11610	0	0	**0**	0	0	**0**	9	9	9	**9.0**	10	9	10	**9.7**
		11612	0	0	**0**	0	0	**0**	8	8	9	**8.3**	8	8	8	**8.0**
		11614	0	0	**0**	0	0	**0**	9	8	9	**8.7**	8	7	8	**7.7**
		11616	0	0	**0**	0	0	**0**	8	8	8	**8.0**	7	8	8	**7.7**
		11618	0	0	**0**	2	3	**2.5**	9	8	8	**8.3**	8	8	9	**8.3**
		11620	0	0	**0**	0	0	**0**	8	8	8	**8.0**	8	8	8	**8.0**
		mean			**0.0**			**0.3**				**8.3**				**8.5**
		SD			**0.0**			**0.8**				**0.5**				**0.7**
T12	negative controles	11622	0	0	**0**	0	0	**0**	0	1	1	**0.7**	0	0	1	**0.3**
		11624	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11626	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11628	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11630	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11632	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11634	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11636	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11638	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		11640	0	0	**0**	0	0	**0**	0	0	0	**0.0**	0	0	0	**0.0**
		mean			**0.0**			**0.0**				**0.1**				**0.0**
		SD			**0.0**			**0.0**				**0.2**				**0.1**
T13	Poly I:C 10 µg	11642	0	0	**0**	0	0	**0**	4	4	4	**4.0**	4	4	4	**4.0**
		11644	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	7	7	**7.0**
		11646	0	0	**0**	0	0	**0**	7	7	8	**7.3**	8	8	8	**8.0**
		11648	0	0	**0**	0	0	**0**	5	4	5	**4.7**	6	6	6	**6.0**
		11650	0	0	**0**	0	0	**0**	5	5	5	**5.0**	6	6	6	**6.0**
		11652	0	0	**0**	0	0	**0**	7	7	7	**7.0**	7	7	7	**7.0**
		11654	0	0	**0**	0	0	**0**	6	6	7	**6.3**	7	7	7	**7.0**
		11656	0	0	**0**	0	0	**0**	4	4	4	**4.0**	5	5	5	**5.0**
		11658	0	0	**0**	0	0	**0**	8	7	8	**7.7**	8	8	8	**8.0**
		mean			**0.0**			**0.0**				**5.8**				**6.4**
		SD			**0.0**			**0.0**				**1.3**				**1.3**
T14	Poly I:C 100 µg	11660	0	0	**0**	0	0	**0**	4	4	4	**4.0**	7	7	7	**7.0**
		11662	0	0	**0**	0	0	**0**	4	4	5	**4.3**	7	7	7	**7.0**
		11664	0	0	**0**	0	0	**0**	5	5	5	**5.0**	7	7	7	**7.0**
		11666	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	7	8	**7.3**
		11668	0	0	**0**	0	0	**0**	6	6	7	**6.3**	8	8	8	**8.0**
		11670	0	0	**0**	0	0	**0**	6	6	6	**6.0**	7	7	8	**7.3**
		11672	0	0	**0**	0	0	**0**	6	6	5	**5.7**	7	8	9	**8.0**
		11674	0	0	**0**	0	0	**0**	6	6	5	**5.7**	8	8	8	**8.0**
		11676	0	0	**0**	1	0	**0.5**	7	7	6	**6.7**	8	8	8	**8.0**
		mean			**0.0**			**0.1**				**5.5**				**7.5**
		SD			**0.0**			**0.2**				**0.8**				**0.4**

Conclusions

[0164] The goal was to study adjuvant activity of three different immune stimulants. This was tested by measuring the serological response after vaccination with oil emulsion vaccines containing a suboptimal concentration of inactivated NDV and different concentrations of one of three different immune stimulants.

[0165] The following immune stimulants were investigated:

ODN1:[CholTEG]-TGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT ("GCGT3-TG4T-5Chol") (SEQ ID NO:1) ([CholTEG]=5'-triethyleneglycol-linked cholesteryl modification),

ODN2:TGGGGTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT ("GCGT3-TG4T") (SEQ ID NO:252),

ODN3:tcgtcgttttgtcgttttgtcgtt ("2006-PTO") (SEQ ID NO:3).

[0166] The backbones of ODN1 and ODN2 immune were phosphodiester-linked, while the backbone of ODN3 was phosphorothioate-linked. The efficacy of each ODN was determined at three different doses; 100 ng, 1000 ng and 5000 ng, supplemented to the suboptimal NDV vaccine.

[0167] The serological response was determined at days 0 (prior to vaccination), 7, 14 and 21 after vaccination to investigate whether the addition of these immune stimulants may also lead to an earlier immune response. On days 0 and 7 post vaccination (pv) no antibody levels against NDV were detected, with the exception of one animal (#11618) in the non-diluted NDV vaccine group at day 7.

[0168] The serological response expressed as Log2 HI titres showed significant differences ($p<0.0001$) between the non-diluted and the suboptimal NDV vaccines at days 14 and 21 pv, indicating that the dilution factor of 50 times was sufficient to create the suboptimal vaccine dose.

[0169] The negative control group remained negative during the entire study, indicating that the immune stimulants without NDV vaccine did not result in a non-specific immune response.

[0170] The positive control Poly I:C 100 μg dose group showed significantly higher HI titres compared to the naïve NDV vaccine at day 21 ($p=0.0053$), indicating that this dose group served as a valid positive control group.

[0171] The GCGT3-TG4T-5Chol (ODN1) group showed significantly higher HI titres when compared to the diluted NDV vaccine at day 14 pv for all three doses; 100 ng ($p=0.0214$), 1000 ng ($p=0.0003$) and 5000 ng ($p=0.0243$). At day 21 pv, however, no significant differences were observed.

[0172] The GCGT3-TG4T (ODN2) group showed significantly higher HI titres when compared to the diluted NDV vaccine at day 14 pv for all three doses; 100 ng (p=0.0003), 1000 ng (p=0.0027) and 5000 ng (p=0.0236). At day 21 significant differences (p=0.0083) were only measured at the 100 ng dose group.

[0173] The 2006-PTO (ODN3) group showed significantly higher HI titres compared to the diluted NDV vaccine at day 14 pv for two doses; 1000 ng (p=0.0081) and 5000 ng (p=0.0059). At day 21 pv significant differences (p=0.0296) were only measured at the 5000 ng dose group.

[0174] In conclusion, the highest mean HI titres were observed with the 100 ng GCGT3-TG4T (ODN2) dose group, 7.1 Log2 (14 days pv) and 7.6 Log2 (21 days pv), indicating an increase in titres when compared to the naïve NDV vaccine of 2.3 Log2 and 1.4 Log2 at day 14 and 21 pv, respectively.

[0175] The titres of the 1000 ng GCGT3-TG4T-5Chol (ODN1) dose group, 6.9 Log2 and 7.3 Log2, at day 14 and 21 pv respectively were almost similar to the ODN2 group. At day 14 pv no significant difference (p=0.7513) between ODN1 and ODN2 groups was observed.

[0176] The titres of the 5000 ng 2006-PTO (ODN3) dose group were 6.2 Log2 and 7.3 Log2 at day 14 and 21 pv, respectively. At day 14 pv, the ODN3 group significantly differed (p=0.0300) from both the ODN1 and ODN2 groups (**FIG. 22** and **FIG. 23**).

[0177] At day 21 pv no significant differences between all ODN groups were shown.

[0178] These results therefore indicate that all ODNs were capable of significantly increasing the serological response, especially on day 14 after vaccination, also indicating an earlier onset of immunity.

EMBODIMENTS

[0179] For futher illustration, additional non-limiting embodiments of the present disclosure are set forth below.

[0180] For example, embodiment 1 is an immunostimulatory composition comprising:

an immunomodulator composition comprising a nucleic acid plasmid and a liposomal delivery vehicle; and

an immunostimulatory oligonucleotide having at least one CpG motif and a guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide.

[0181] Embodiment 2 is the immunostimulatory composition of embodiment 1, wherein the immunomodulator composition and the immunostimulatory oligonucleotide are present in synergistically effective amounts.

[0182] Embodiment 3 is the immunostimulatory composition of embodiment 1 or 2, wherein the immunostimulatory oligonucleotide comprises a ligand for a cytosolic nucleic acid surveillance molecule.

[0183] Embodiment 4 is the immunostimulatory composition of embodiment 3, wherein the immune cytosolic nucleic acid surveillance molecule is a toll-like receptor (TLR).

[0184] Embodiment 5 is the immunostimulatory composition of embodiment 3 or 4, wherein the cytosolic nucleic acid surveillance molecule is TLR21.

[0185] Embodiment 6 is the immunostimulatory composition of any one of the preceding embodiments, wherein the immunomodulator composition has a nucleic acid concentration of about 200 μg/ml.

[0186] Embodiment 7 is the immunostimulatory composition of any one of the preceding embodiments, wherein the concentration of the immunostimulatory oligonucleotide is between about 10 μM and 0.5 μM.

[0187] Embodiment 8 is the immunostimulatory composition of embodiment 7, wherein the concentration of the immunostimulatory oligonucleotide is about 2 μM.

[0188] Embodiment 9 is the immunostimulatory composition of any one of the preceding embodiments, wherein the nucleic acid plasmid concentration of the immunomodulator composition is greater than the concentration of the immunostimulatory oligonucleotide.

[0189] Embodiment 10 is the immunostimulatory composition of any one of the preceding embodiments, wherein the immunomodulator composition is present in non-cytotoxic amounts.

[0190] Embodiment 11 is the immunostimulatory composition of any one of the preceding embodiments further comprising a pharmaceutical carrier.

[0191] Embodiment 12 is the immunostimulatory composition of any one of the preceding embodiments, wherein the liposomal delivery vehicle comprises multilamellar vesicle lipids, extruded lipids, or both.

[0192] Embodiment 13 is the immunostimulatory composition of any one of the preceding embodiments, wherein the liposomal delivery vehicle is cationic.

[0193] Embodiment 14 is the immunostimulatory composition of embodiment 13, wherein the cationic liposomal delivery vehicle comprises pairs of lipids selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and cholesterol; N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol; 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM) and cholesterol; and dimethyldioctadecylammonium bromide (DDAB) and cholesterol.

[0194] Embodiment 15 is the immunostimulatory composition of any one of the preceding embodiments, wherein the nucleic acid plasmid is non-coding.

[0195] Embodiment 16 is the immunostimulatory composition of any one of the preceding embodiments, wherein the nucleic acid plasmid is bacterially derived.

[0196] Embodiment 17 is the immunostimulatory composition of any one of the preceding embodiments, wherein the nucleic acid plasmid is immunogenic.

[0197] Embodiment 18 is the immunostimulatory composition of any one of embodiments 1-17, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO. 265.

[0198] Embodiment 19 is the immunostimulatory composition of any one of embodiments 1-17, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO.:266.

[0199] Embodiment 20 is the immunostimulatory composition of any one of embodiments 1-17, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:268.

[0200] Embodiment 21 is the immunostimulatory composition of any one of the preceding embodiments, wherein the guanine nucleotide enriched sequence comprises a first plurality of guanine nucleotides.

[0201] Embodiment 22 is the immunostimulatory composition of embodiment 21, wherein the first plurality of guanine nucleotides comprises three to eight guanine nucleotides.

[0202] Embodiment 23 is the immunostimulatory composition of embodiment 21 or 22, wherein the immunostimulatory oligonucleotide comprises SEQ ID NO:16, 17, 18, 19, 20, 21, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 77, 78, 81, 82, 85, 86, 89, 90, 92, 93, 96, 97, 100, 102, 104, 106, 108, 143, or 1.

[0203] Embodiment 24 is the immunostimulatory composition of embodiment 21 or 22 further comprising a second plurality of guanine nucleotides downstream from the first plurality of guanine nucleotides.

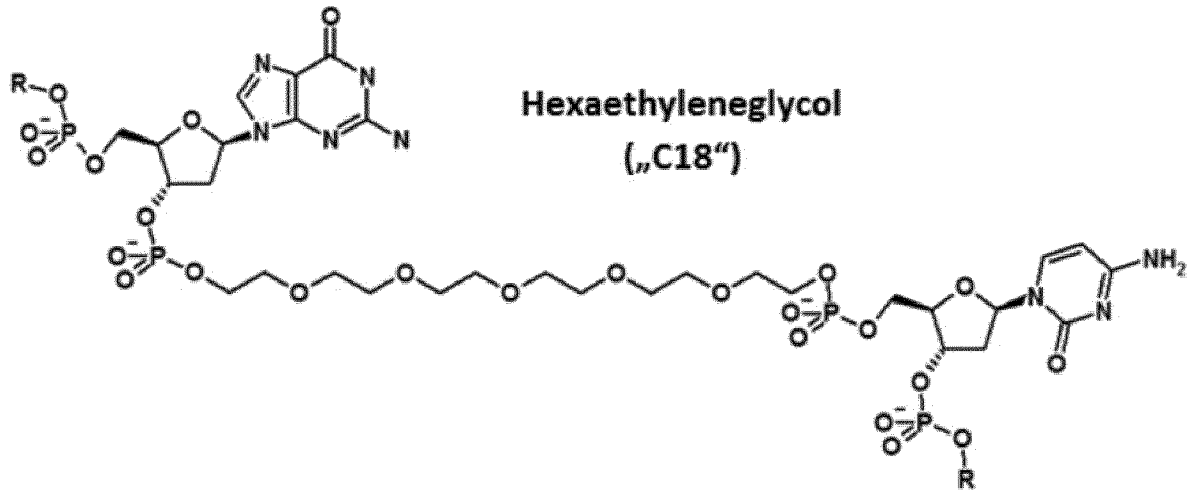
[0204] Embodiment 25 is the immunostimulatory composition of embodiment 24, wherein the immunostimulatory oligonucleotide comprises SEQ ID NO:141, 142, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, or GCGT-Gwire3.

[0205] Embodiment 26 is the immunostimulatory compositions of embodiment 24 or 25 wherein the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least two nucleotides.

[0206] Embodiment 27 is the immunostimulatory compositions of any one of embodiments 21 to 26, wherein the first plurality of guanine nucleotides and the at least one CpG motif is separated by at least 3 nucleotides.

[0207] Embodiment 28 is the immunostimulatory compositions of embodiments 21 to 27, wherein the first plurality of guanine nucleotides and the at least one CpG motif is separated by a hexaethyleneglycol, tetraethyleneglycol, propanediol, or derivatives thereof.

[0208] Embodiment 29 is the immunostimulatory composition of embodiment 28, wherein the structure of the hexaethyleneglycol is:



[0209] Embodiment 30 is the immunostimulatory composition of any one of the preceding embodiments, wherein the immunostimulatory oligonucleotide further comprises a plurality of CpG motifs, each CpG motif of the plurality of CpG motifs being separated from the others of the plurality of CpG motifs by a spacer.

[0210] Embodiment 31 is the immunostimulatory composition of embodiment 30, wherein the spacer comprises at least one nucleotide or nucleotide analog.

[0211] Embodiment 32 is the immunostimulatory composition of embodiment 31, wherein the spacer comprises a deoxyribosephosphate bridge.

[0212] Embodiment 33 is the immunostimulatory composition of embodiment 32, wherein the deoxyribosephosphate bridge is abasic.

[0213] Embodiment 34 is the immunostimulatory composition of embodiment 31, wherein the spacer comprises a carbon chain.

[0214] Embodiment 35 is the immunostimulatory composition of embodiment 34, wherein the carbon chain is derived from 1,3-propanediol.

[0215] Embodiment 36 is the immunostimulatory composition of embodiment 31, wherein the spacer comprises a repeated chemical unit.

[0216] Embodiment 37 is the immunostimulatory composition of embodiment 36, wherein the repeated chemical unit is an ethylene glycol.

[0217] Embodiment 38 is the immunostimulatory composition of any one of the preceding embodiments, wherein the immunostimulatory oligonucleotide comprises at least one nucleotide analog.

[0218] Embodiment 39 is the immunostimulatory composition of any one of the preceding embodiments, wherein the immunostimulatory oligonucleotide further comprises a phosphodiester backbone.

[0219] Embodiment 40 is the immunostimulatory composition of any embodiments 1-38, wherein the immunostimulatory oligonucleotide further comprises a phosphorothioate backbone.

[0220] Embodiment 41 is the immunostimulatory composition of any one of the preceding embodiments, wherein the immunostimulatory oligonucleotide comprises a lipid moiety.

[0221] Embdoiment 42 is the immunostimulatory composition of embodiment 41, wherein the lipid moiety is cholesteryl.

[0222] Embodiment 43 is the immunostimulatory composition of any one of embodiments 41 to 42, wherein the lipid moiety is at or near the 5' terminus of the immunostimulatory oligonucleotide.

[0223] Embodiment 44 is the immunostimulatory composition of any one of the preceding embodiments, comprising a CpG sequence element at a 5' terminus, a 3' terminus, or both.

[0224] Embodiment 45 is the immunostimulatory composition of embodiment 44 having at least two CpG sequence elements.

[0225] Embodiment 46 is the immunostimulatory composition of embodiment 44 or 45, wherein the CpG sequence elements are GCGA, GCGG, ACGC, CCGC, GCGT, or TCGC.

[0226] Embodiment 47 is a method of preparing the immunostimulatory composition of any one of the preceding claims comprising:

combining the immunomodulator composition and the immunostimulatory oligonucleotide, to form an immunostimulatory composition;

centrifuging the immunostimulatory composition to generate a supernatant and a pellet; and

isolating the pellet.

[0227] Embodiment 48 is a method for stimulating toll-like receptor 21 (TLR21) comprising:

administering an immunostimulatory oligonucleotide and an immunomodulator composition, wherein the immunostimulatory oligonucleotide has at least one CpG motif and a guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide, and wherein the immunomodulator composition comprises a noncoding nucleic acid plasmid and a lipid delivery vehicle.

[0228] Embodiment 49 is the method of embodiment 48, wherein the immunomodulator composition and the immunostimulatory oligonucleotide are present in synergistically effective amounts.

[0229] Embodiment 50 is the method of embodiment 48 or 49, wherein the immunostimulatory oligonucleotide comprises a ligand for TLR21.

[0230] Embodiment 51 is the method of any one of embodiments 48 to 50, wherein the nucleic acid plasmid concentration of the immunomodulator composition is about 200 μg/ml.

[0231] Embodiment 52 is the method of any one of embodiments 48 to 51, wherein the concentration of the immunostimulatory oligonucleotide is between about 10 μM and 0.5 μM.

[0232] Embodiment 53 is the method of embodiment 52, wherein the concentration of the immunostimulatory oligonucleotide is about 2 μM.

[0233] Embodiment 54 is the method of any one of embodiments 48 to 53, wherein the nucleic acid plasmid concentration of the immunomodulator composition is greater than the concentration of the immunostimulatory oligonucleotide.

[0234] Embodiment 55 is the method of any one of embodiments 48 to 54, wherein the immunomodulator composition is present in non-cytotoxic amounts.

[0235] Embodiment 56 is the method of any one of embodiments 48 to 55, wherein the immunomodulator composition further comprises a pharmaceutical carrier.

[0236] Embodiment 57 is the method of any one of embodiments 48 to 56, wherein the liposomal delivery vehicle comprises multilamellar vesicle lipids, extruded lipids, or both.

[0237] Embodiment 58 is the method of any one of embodiments 48 to 57, wherein the liposomal delivery vehicle is cationic.

[0238] Embodiment 59 is the method of embodiment 58, wherein the cationic liposomal delivery vehicle comprises pairs of lipids selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and cholesterol; N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol; 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM) and cholesterol; and dimethyldioctadecylammonium bromide (DDAB) and cholesterol.

[0239] Embodiment 60 is the method of any one of embodiment s 48 to 59, wherein the nucleic acid plasmid is non-coding.

[0240] Embodiment 61 is the method of any of embodiments 48 to 60, wherein the nucleic acid plasmid is bacterially derived.

[0241] Embodiment 62 is the method of any one of embodiments 48 to 61, wherein the nucleic acid plasmid is immunogenic.

[0242] Embodiment 63 is the method of any one of embodiments 48 to 62, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:265.

[0243] Embodiment 64 is the method of any one of embodiments 48 to 62, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:266.

[0244] Embodiment 65 is the method of any one of embodiments 48 to 62, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:268.

[0245] Embodiment 66 is the method of any one of embodiments 48 to 65, wherein the guanine nucleotide enriched sequence comprises a first plurality of guanine nucleotides.

[0246] Embodiment 67 is the method of embodiment 66, wherein the first plurality of guanine nucleotides comprises three to eight guanine nucleotides.

[0247] Embodiment 68 is the method of embodiment 66 or 67, wherein the immunostimulatory oligonucleotide comprises SEQ ID NO:1, 16, 17, 18, 19, 20, 21, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 77, 78, 81, 82, 85, 86, 89, 90, 92, 93, 96, 97, 100, 102, 104, 106, 108, or 143.

[0248] Embodiment 69 is the method of any one of embodiments 48-68, wherein the immunostimulatory oligonucleotide further comprises a second plurality of guanine nucleotides downstream from the first plurality of guanine nucleotides.

[0249] Embodiment 70 is the method of embodiment 69, wherein the immunostimulatory oligonucleotide comprises SEQ ID NO:141, 142, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, or GCGT-Gwire3.

[0250] Embodiment 71 is the method of embodiment 69 or 70, wherein the first plurality of guanine nucleotides, the second plurality of guanine nucleotides, or both facilitate the formation of quaternary structures *in vitro*, *in vivo*, or both.

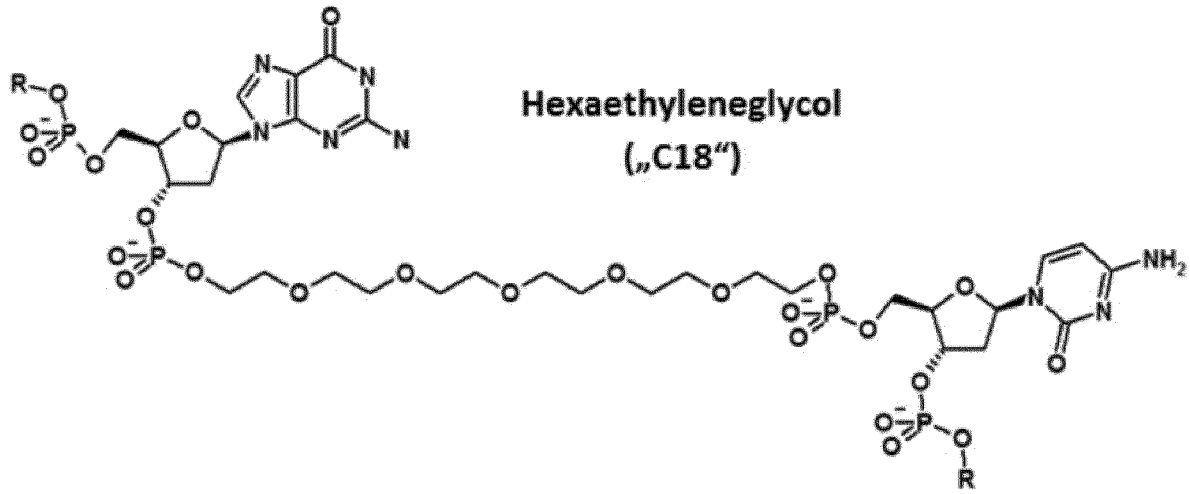
[0251] Embodiment 72 is the method of embodiment 70 or 71, wherein the first and second pluralities of guanine nucleotides facilitate formation of quaternary structures having a G-wire conformation.

[0252] Embodiment 73 is the method of any one of embodiments 70 to 72, wherein the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least two nucleotides.

[0253] Embodiment 74 is the method of any one of embodiments 67 to 73, wherein the first plurality of guanine nucleotides and the at least one CpG motif are separated by at least 3 nucleotides.

[0254] Embodiment 75 is the method of any one of embodiments 67 to 73, wherein the first plurality of guanine nucleotides and the at least one CpG motif are separated by a hexaethyleneglycol.

[0255] Embodiment 76 is the method of embodiment 75, wherein the structure of the hexaethyleneglycol is:



[0256] Embodiment 77 is the method of any one of embodiments 48 to 76, wherein the immunostimulatory oligonucleotide further comprises a plurality of CpG motifs, each CpG motif of the plurality of CpG motifs being separated by a spacer.

[0257] Embodiment 78 is the method of embodiment 77, wherein the spacer comprises at least one nucleotide or nucleotide derivative.

[0258] Embodiment 79 is the method of embodiment 78, wherein the spacer is a deoxyribosephosphate bridge.

[0259] Embodiment 80 is the method of embodiment 79, wherein the deoxyribosephosphate bridge is abasic.

[0260] Embodiment 81 is the method of embodiment 78, wherein the spacer comprises a carbon chain.

[0261] Embodiment 82 is the method of embodiment 81, wherein the carbon chain is derived from 1,3-propanediol.

[0262] Embodiment 83 is the method of embodiment 78, wherein the spacer comprises a repeated chemical unit.

[0263] Embodiment 84 is the method of embodiment 83, wherein the repeated chemical unit is an ethylene glycol.

[0264] Embodiment 85 is the method of any one of the embodiments 48 to 84, wherein the immunomodulator composition, the immunostimulatory oligonucleotide, or both further comprise at least one nucleotide analog.

[0265] Embodiment 86 is the method of any one of embodiments 48 to 85, wherein the immunostimulatory oligonucleotide comprises a phosphodiester backbone.

[0266] Embodiment 87 is the method of any one of embodiments 48 to 85, wherein the immunostimulatory oligonucleotide comprises a phosphorothioate backbone.

[0267] Embodiment 88 is the method of any one of embodiments 48 to 87, wherein the immunostimulatory oligonucleotide further comprises a lipid moiety.

[0268] Embodiment 89 is the method of embodiment 88, wherein the lipid moiety enhances bioavailability of the immunostimulatory oligonucleotide.

[0269] Embodiment 90 is the method of embodiment 88 or 89, wherein the lipid moiety is cholesteryl.

[0270] Embodiment 91 is the method of any one of embodiments 88 to 90, wherein the lipid moiety is at or near the 5' terminus of the immunostimulatory oligonucleotide.

[0271] Embodiment 92 is the method of any one of embodiments 48 to 91, comprising a CpG sequence element at a 5' terminus, a 3' terminus, or both.

[0272] Embodiment 93 is the method of embodiment 92 having at least two CpG sequence elements.

[0273] Embodiment 94 is the method of embodiment 92 or 93, wherein the CpG sequence elements are GCGA, GCGG, ACGC, CCGC, GCGT, or TCGC.

[0274] Embodiment 95 is the methods of any one of embodiments 48 to 94, wherein the immunomodulator composition and the immunostimulatory oligonucleotide are administered simultaneously.

[0275] Embodiment 96 is a method of eliciting an immune response in a subject comprising administering to the subject the immunostimulatory composition of any one of embodiments 1 to 46.

[0276] Embodiment 97 is an immunostimulatory composition comprising:

a nucleic acid plasmid and a liposomal delivery vehicle; and

an immunostimulatory oligonucleotide comprising SEQ ID NO:1.

[0277] Embodiment 98 is the immunostimulatory composition of embodiment 97, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:265.

[0278] Embodiment 99 is the immunostimulatory composition of embodiment 97, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:266.

[0279] Embodiment 100 is the immunostimulatory composition of embodiment 97, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:268.

[0280] Embodiment 101 is the immunostimulatory composition of any one of embodiments 97-100, wherein the liposomal delivery vehicle comprises multilamellar vesicle lipids, extruded lipids, or both.

[0281] Embodiment 102 is the immunostimulatory composition of embodiment 101, wherein the liposomal delivery vehicle is cationic.

[0282] Embodiment 103 is the immunostimulatory composition of embodiment 102, wherein the cationic liposomal delivery vehicle comprises pairs of lipids selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and cholesterol; N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol; 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM) and cholesterol; and dimethyldioctadecylammonium bromide (DDAB) and cholesterol.

[0283] Embodiment 104 is the immunostimulatory composition of any one of embodiments 97-103, wherein the immunostimulatory oligonucleotide further comprises a 5' cholesteryl modification.

[0284] Embodiment 105 is the immunostimulatory composition of embodiment 104, wherein the 5' cholesteryl modification comprises a triethyleneglycol linker.

[0002] When introducing elements of the present disclosure or the preferred embodiment(s) thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[0003] In view of the above, it will be seen that the several objects of the discxlosure are achieved and other advantageous results attained.

[0004] As various changes could be made in the above products and methods without departing from the scope of the disclosure, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

What is claimed:

1. An immunostimulatory composition comprising:

a) an immunomodulator composition comprising a nucleic acid plasmid and a liposomal delivery vehicle; and

b) an immunostimulatory oligonucleotide having at least one CpG motif and a guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide.

2. The immunostimulatory composition of claim 1, wherein the immunomodulator composition and the immunostimulatory oligonucleotide are present in synergistically effective amounts.

3. The immunostimulatory composition of claim 1 or 2, wherein the immunostimulatory oligonucleotide comprises a ligand for a cytosolic nucleic acid surveillance molecule.

4. The immunostimulatory composition of claim 3, wherein the immune cytosolic nucleic acid surveillance molecule is a toll-like receptor (TLR).

5. The immunostimulatory composition of claim 3 or 4, wherein the cytosolic nucleic acid surveillance molecule is TLR21.

6. The immunostimulatory composition of any one of the preceding claims, wherein the nucleic acid plasmid concentration of the immunomodulator composition is greater than the concentration of the immunostimulatory oligonucleotide.

7. The immunostimulatory composition of any one of the preceding claims further comprising a pharmaceutical carrier.

8. The immunostimulatory composition of any one of the preceding claims, wherein the liposomal delivery vehicle comprises multilamellar vesicle lipids, extruded lipids, or both.

9. The immunostimulatory composition of any one of the preceding claims, wherein the liposomal delivery vehicle is cationic.

10. The immunostimulatory composition of claim 8, wherein the cationic liposomal delivery vehicle comprises pairs of lipids selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and cholesterol; N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) and cholesterol; 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM) and cholesterol; and dimethyldioctadecylammonium bromide (DDAB) and cholesterol.

11. The immunostimulatory composition of any one of the preceding claims, wherein the nucleic acid plasmid is non-coding.

12. The immunostimulatory composition of any one of claims 1-11, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO. 265.

13. The immunostimulatory composition of any one of claims 1-11, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO.:266.

14. The immunostimulatory composition of any one of claims 1-11, wherein the nucleic acid plasmid has at least 75% sequence identity with SEQ ID NO:268.

15. The immunostimulatory composition of any one of the preceding claims, wherein the guanine nucleotide enriched sequence comprises a first plurality of guanine nucleotides.

16. The immunostimulatory composition of claim 15, wherein the first plurality of guanine nucleotides comprises three to eight guanine nucleotides.

17. The immunostimulatory composition of claim 15 or 16, wherein the immunostimulatory oligonucleotide comprises SEQ ID NO:16, 17, 18, 19, 20, 21, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 77, 78, 81, 82, 85, 86, 89, 90, 92, 93, 96, 97, 100, 102, 104, 106, 108, 143, or 1.

18. The immunostimulatory composition of claim 15 or 16 further comprising a second plurality of guanine nucleotides downstream from the first plurality of guanine nucleotides.

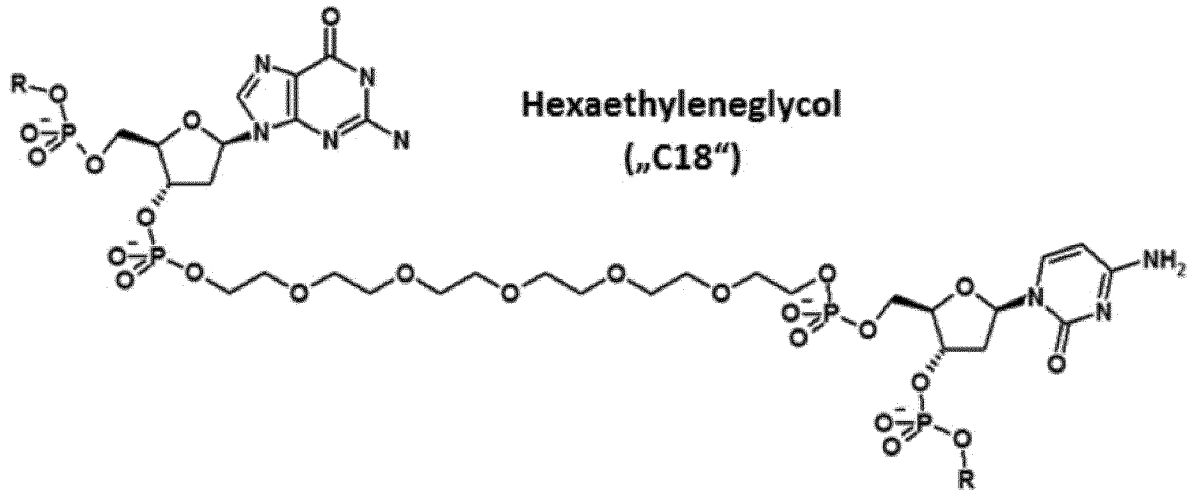
19. The immunostimulatory composition of claim 18, wherein the immunostimulatory oligonucleotide comprises SEQ ID NO:141, 142, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, or GCGT-Gwire3.

20. The immunostimulatory compositions of claim 18 or 19 wherein the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least two nucleotides.

21. The immunostimulatory compositions of any one of claims 15 to 20, wherein the first plurality of guanine nucleotides and the at least one CpG motif is separated by at least 3 nucleotides.

22. The immunostimulatory compositions of claims 15 to 21, wherein the first plurality of guanine nucleotides and the at least one CpG motif is separated by a hexaethyleneglycol, tetraethyleneglycol, propanediol, or derivatives thereof.

23. The immunostimulatory composition of claim 22, wherein the structure of the hexaethyleneglycol is:



24. The immunostimulatory composition of any one of the preceding claims, wherein the immunostimulatory oligonucleotide further comprises a plurality of CpG motifs, each CpG motif of the plurality of CpG motifs being separated from the others of the plurality of CpG motifs by a spacer.

25. The immunostimulatory composition of claim 24, wherein the spacer comprises at least one nucleotide or nucleotide analog.

26. The immunostimulatory composition of claim 25, wherein the spacer comprises a deoxyribosephosphate bridge.

27. The immunostimulatory composition of claim 26, wherein the deoxyribosephosphate bridge is abasic.

28. The immunostimulatory composition of claim 25, wherein the spacer comprises a carbon chain.

29. The immunostimulatory composition of claim 28, wherein the carbon chain is derived from 1,3-propanediol.

30. The immunostimulatory composition of claim 25, wherein the spacer comprises a repeated chemical unit.

31. The immunostimulatory composition of claim 30, wherein the repeated chemical unit is an ethylene glycol.

32. The immunostimulatory composition of any claims 1-31, wherein the immunostimulatory oligonucleotide further comprises a phosphorothioate backbone.

33. The immunostimulatory composition of any one of the preceding claims, wherein the immunostimulatory oligonucleotide comprises a lipid moiety.

34. The immunostimulatory composition of claim 33, wherein the lipid moiety is cholesteryl.

35. The immunostimulatory composition of any one of claims 33 to 34, wherein the lipid moiety is at or near the 5' terminus of the immunostimulatory oligonucleotide.

36. The immunostimulatory composition of any one of the preceding claims, comprising a CpG sequence element at a 5' terminus, a 3' terminus, or both.

37. The immunostimulatory composition of claim 36 having at least two CpG sequence elements.

38. The immunostimulatory composition of claim 36 or 37, wherein the CpG sequence elements are GCGA, GCGG, ACGC, CCGC, GCGT, or TCGC.

39. A method of preparing the immunostimulatory composition of any one of the preceding claims comprising:

combining the immunomodulator composition and the immunostimulatory oligonucleotide, to form an immunostimulatory composition;

centrifuging the immunostimulatory composition to generate a supernatant and a pellet; and

isolating the pellet.

40. A method for stimulating toll-like receptor 21 (TLR21) comprising:

administering an immunostimulatory oligonucleotide and an immunomodulator composition, wherein the immunostimulatory oligonucleotide has at least one CpG motif and a guanine nucleotide enriched sequence at or near the 5' terminus of the immunostimulatory oligonucleotide, and wherein the immunomodulator composition comprises a noncoding nucleic acid plasmid and a lipid delivery vehicle.

41. The method of claim 40, wherein the immunomodulator composition and the immunostimulatory oligonucleotide are present in synergistically effective amounts.

42. The method of claim 40 or 41, wherein the immunostimulatory oligonucleotide comprises a ligand for TLR21.

43. A method of eliciting an immune response in a subject comprising administering to the subject the immunostimulatory composition of any one of claims 1 to 38.

44. An immunostimulatory composition comprising:

a. a nucleic acid plasmid and a liposomal delivery vehicle; and

b. an immunostimulatory oligonucleotide comprising SEQ ID NO:1.

45. The immunostimulatory composition of claim 44, wherein the nucleic acid plasmid has at least 75% sequence identity with a sequence selected from the group consisting of SEQ ID NO:265, SEQ ID NO:266, SEQ ID NO:268.

46. The immunostimulatory composition of any one of claims 44-45, wherein the immunostimulatory oligonucleotide further comprises a 5' cholesteryl modification.

47. The immunostimulatory composition of claim 46, wherein the 5' cholesteryl modification comprises a triethyleneglycol linker.

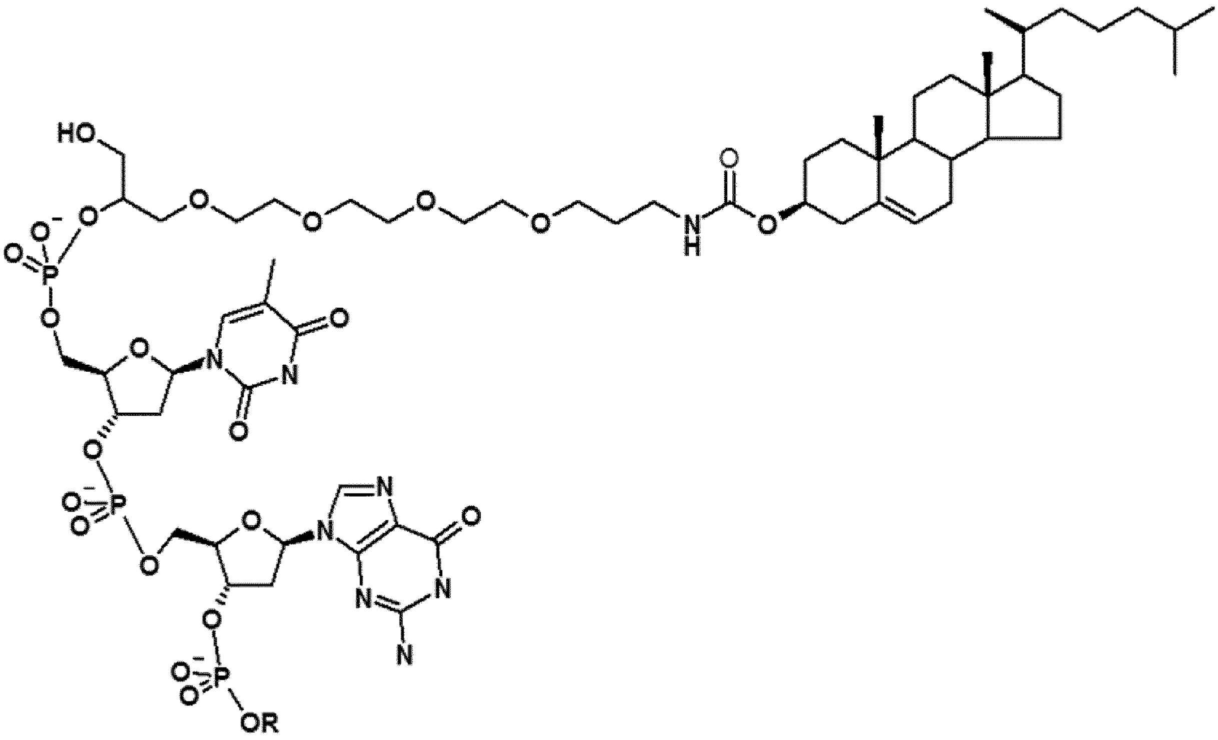


FIG. 1

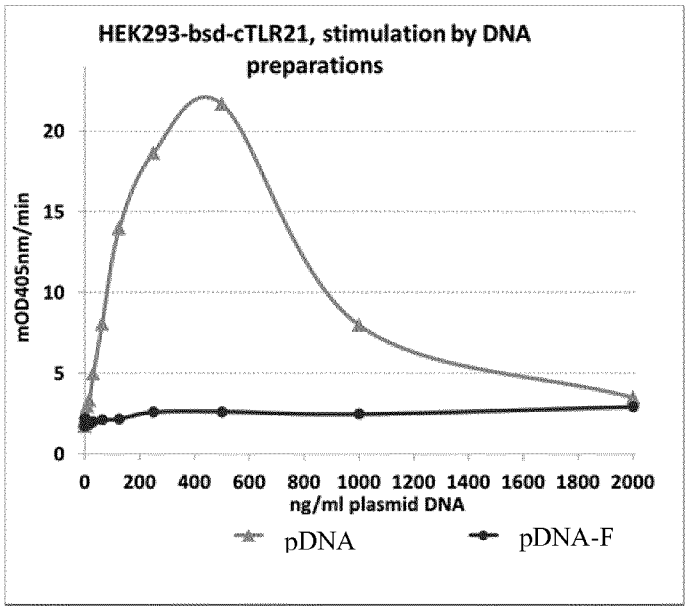

HEK293-bsd-cTLR21, stimulation by DNA preparations
mOD405nm/min
0
5
10
15
20
0 200 400 600 800 1000 1200 1400 1600 1800 2000
ng/ml plasmid DNA
pDNA
pDNA-F

FIG. 2A

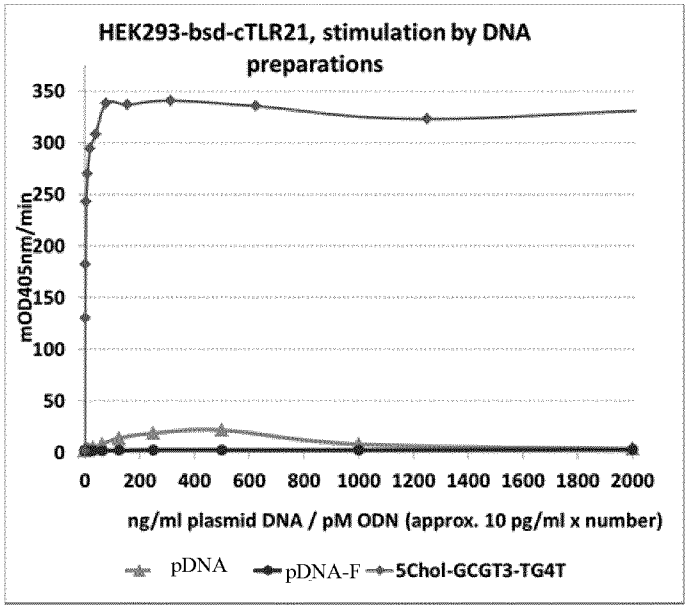

HEK293-bsd-cTLR21, stimulation by DNA preparations
mOD405nm/min
0
50
100
150
200
250
300
350
0 200 400 600 800 1000 1200 1400 1600 1800 2000
ng/ml plasmid DNA / pM ODN (approx. 10 pg/ml x number)
pDNA
pDNA-F
5Chol-GCGT3-TG4T

FIG. 2B

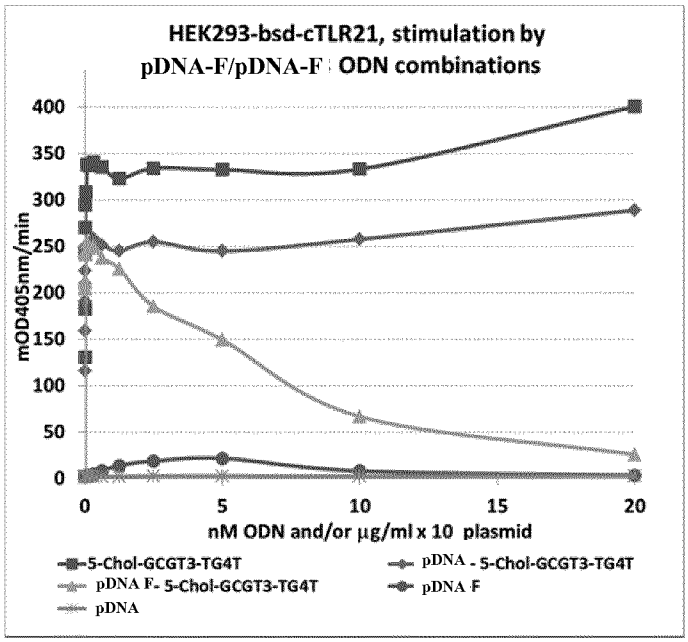

HEK293-bsd-cTLR21, stimulation by
pDNA-F/pDNA-F ODN combinations
mOD405nm/min
400
350
300
250
200
150
100
50
0
0
5
10
15
20
nM ODN and/or µg/ml x 10 plasmid
5-Chol-GCGT3-TG4T
pDNA - 5-Chol-GCGT3-TG4T
pDNA F- 5-Chol-GCGT3-TG4T
pDNA F
pDNA

FIG. 3A

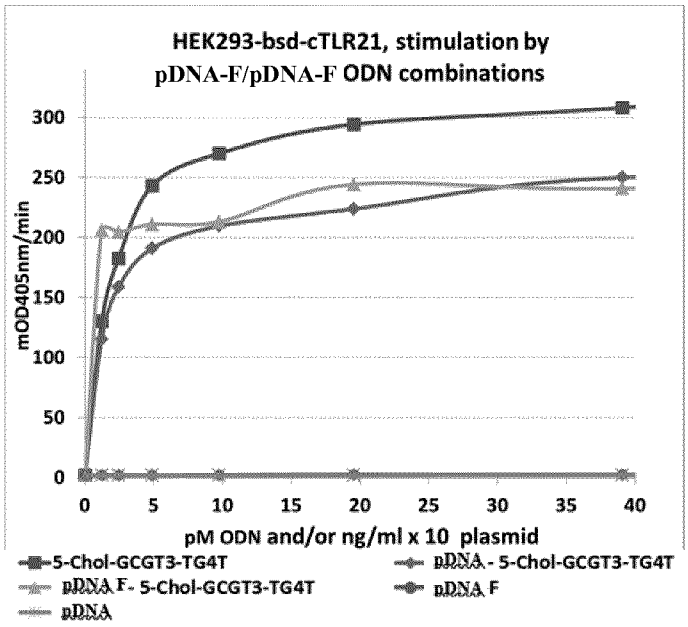

HEK293-bsd-cTLR21, stimulation by
pDNA-F/pDNA-F ODN combinations
mOD405nm/min
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
40
pM ODN and/or ng/ml x 10 plasmid
5-Chol-GCGT3-TG4T
pDNA - 5-Chol-GCGT3-TG4T
pDNA F- 5-Chol-GCGT3-TG4T
pDNA F
pDNA

FIG. 3B

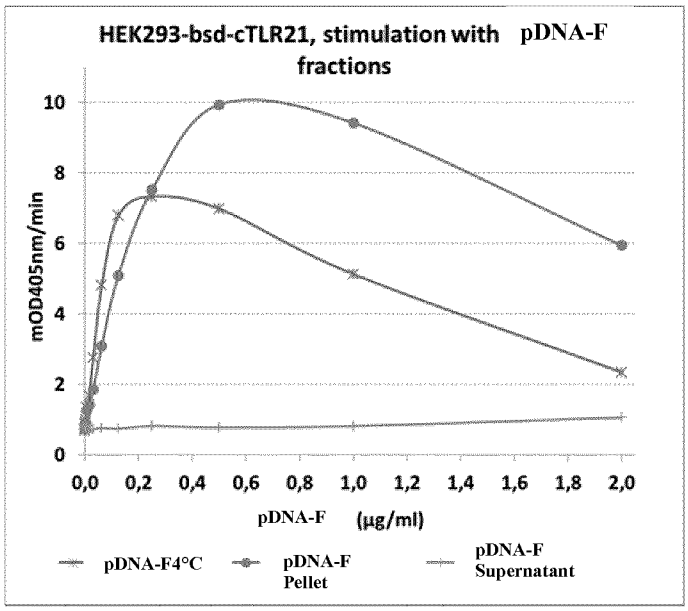

HEK293-bsd-cTLR21, stimulation with pDNA-F fractions
mOD405nm/min
10
8
6
4
2
0
0,0 0,2 0,4 0,6 0,8 1,0 1,2 1,4 1,6 1,8 2,0
pDNA-F (µg/ml)
pDNA-F4°C
pDNA-F Pellet
pDNA-F Supernatant

FIG. 4

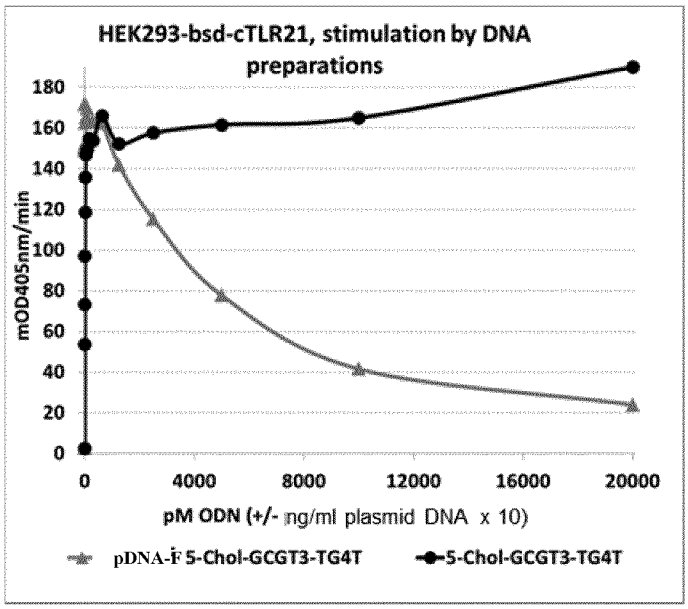

HEK293-bsd-cTLR21, stimulation by DNA preparations
mOD405nm/min
180
160
140
120
100
80
60
40
20
0
0
4000
8000
12000
16000
20000
pM ODN (+/- ng/ml plasmid DNA x 10)
pDNA-F
5-Chol-GCGT3-TG4T
5-Chol-GCGT3-TG4T

FIG. 5A

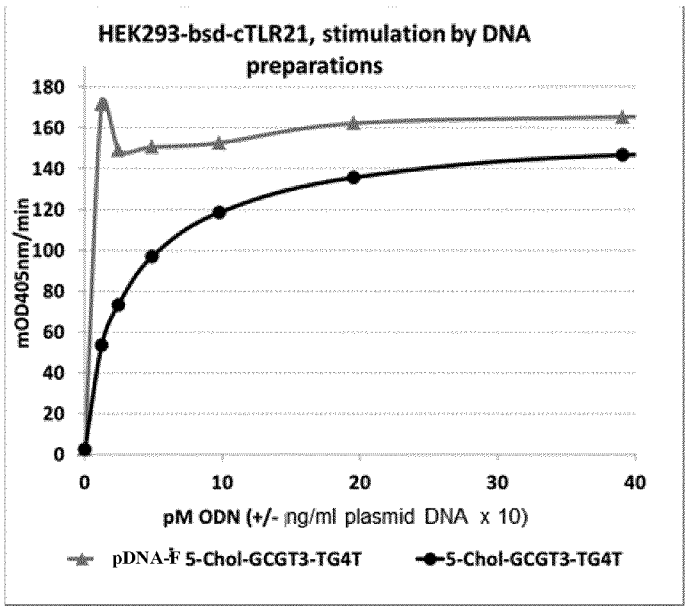

HEK293-bsd-cTLR21, stimulation by DNA preparations
mOD405nm/min
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
pM ODN (+/- ng/ml plasmid DNA x 10)
pDNA-F
5-Chol-GCGT3-TG4T
5-Chol-GCGT3-TG4T

FIG. 5B

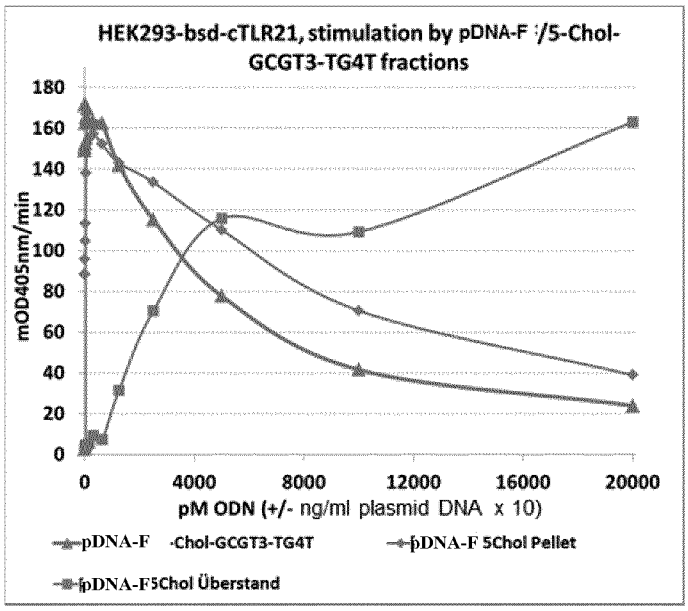

HEK293-bsd-cTLR21, stimulation by pDNA-F /5-Chol-GCGT3-TG4T fractions
mOD405nm/min
180
160
140
120
100
80
60
40
20
0
0
4000
8000
12000
16000
20000
pM ODN (+/- ng/ml plasmid DNA x 10)
pDNA-F
pDNA-F 5Chol-GCGT3-TG4T
pDNA-F 5Chol Pellet
pDNA-F5Chol Überstand

FIG. 6A

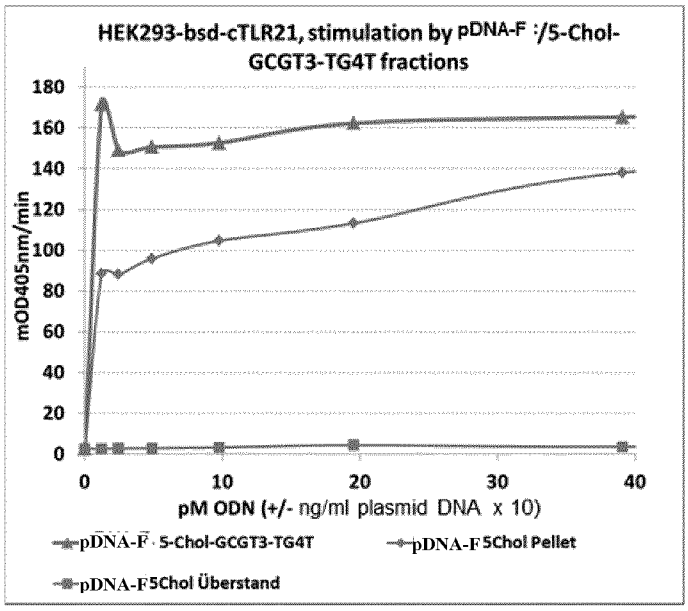

HEK293-bsd-cTLR21, stimulation by pDNA-F /5-Chol-GCGT3-TG4T fractions
mOD405nm/min
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
pM ODN (+/- ng/ml plasmid DNA x 10)
pDNA-F
pDNA-F 5-Chol-GCGT3-TG4T
pDNA-F 5Chol Pellet
pDNA-F5Chol Überstand

FIG. 6B

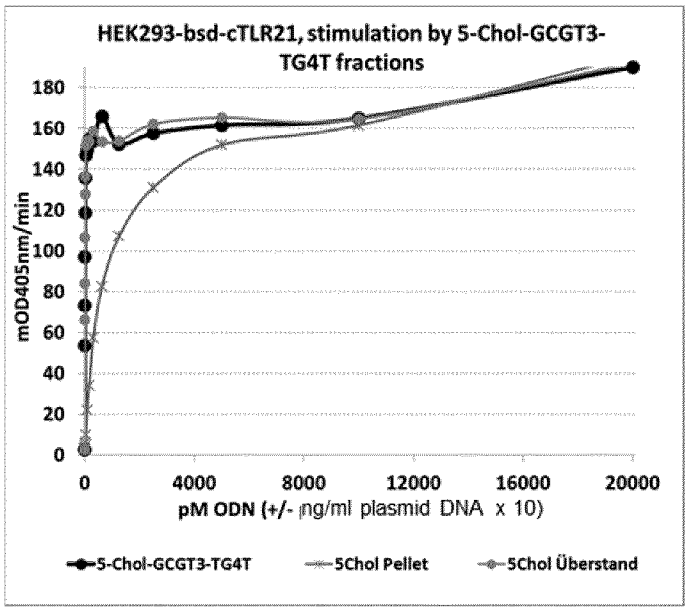

HEK293-bsd-cTLR21, stimulation by 5-Chol-GCGT3-TG4T fractions
mOD405nm/min
180
160
140
120
100
80
60
40
20
0
0
4000
8000
12000
16000
20000
pM ODN (+/- ng/ml plasmid DNA x 10)
5-Chol-GCGT3-TG4T
5Chol Pellet
5Chol Überstand

FIG. 7A

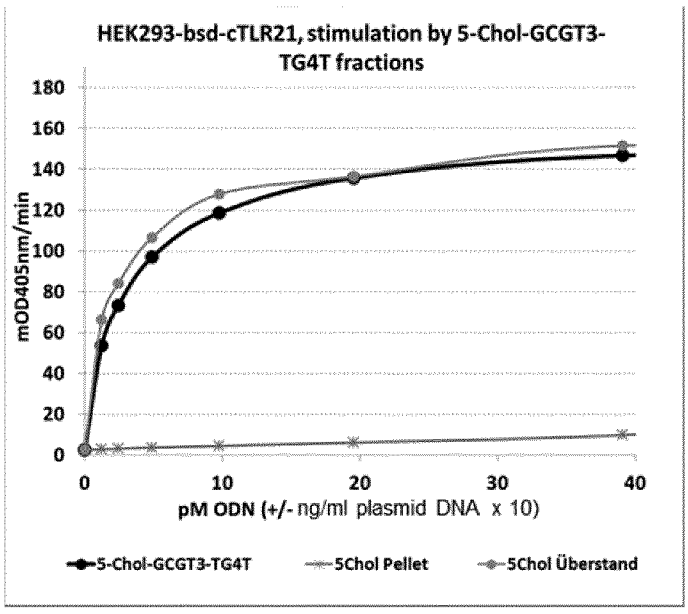

HEK293-bsd-cTLR21, stimulation by 5-Chol-GCGT3-TG4T fractions
mOD405nm/min
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
pM ODN (+/- ng/ml plasmid DNA x 10)
5-Chol-GCGT3-TG4T
5Chol Pellet
5Chol Überstand

FIG. 7B

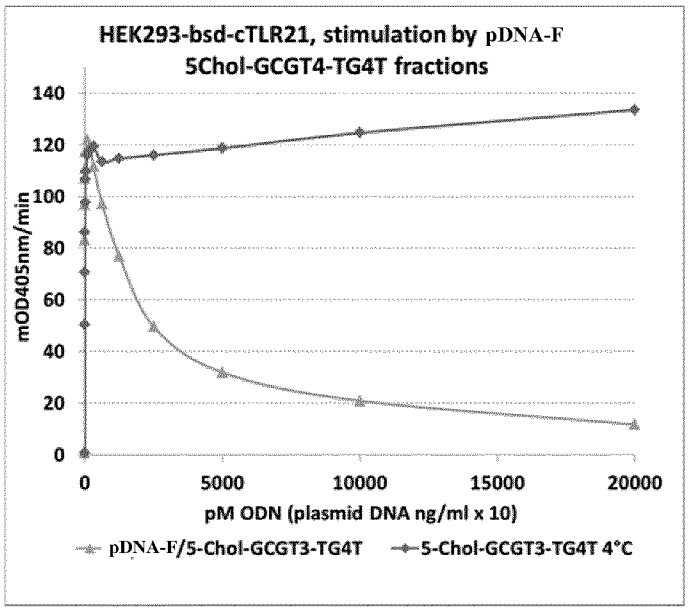

HEK293-bsd-cTLR21, stimulation by pDNA-F 5Chol-GCGT4-TG4T fractions
mOD405nm/min
140
120
100
80
60
40
20
0
0
5000
10000
15000
20000
pM ODN (plasmid DNA ng/ml x 10)
pDNA-F/5-Chol-GCGT3-TG4T
5-Chol-GCGT3-TG4T 4°C

FIG. 8A

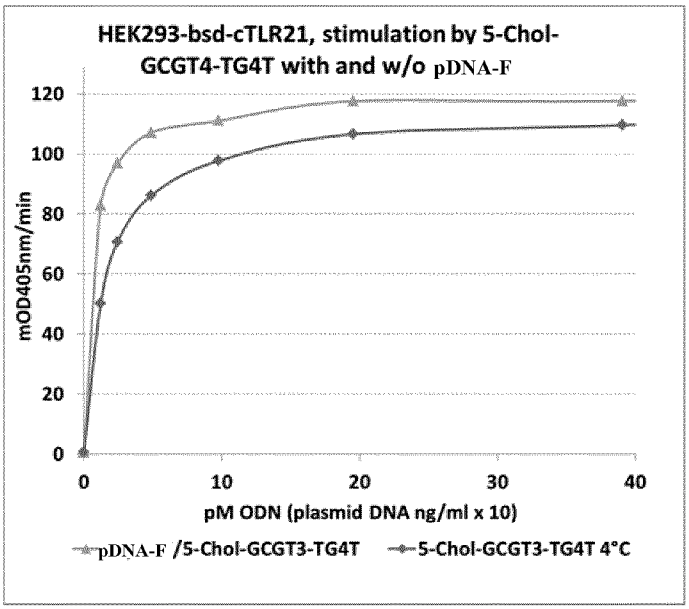

HEK293-bsd-cTLR21, stimulation by 5-Chol-GCGT4-TG4T with and w/o pDNA-F
mOD405nm/min
120
100
80
60
40
20
0
0
10
20
30
40
pM ODN (plasmid DNA ng/ml x 10)
pDNA-F /5-Chol-GCGT3-TG4T
5-Chol-GCGT3-TG4T 4°C

FIG. 8B

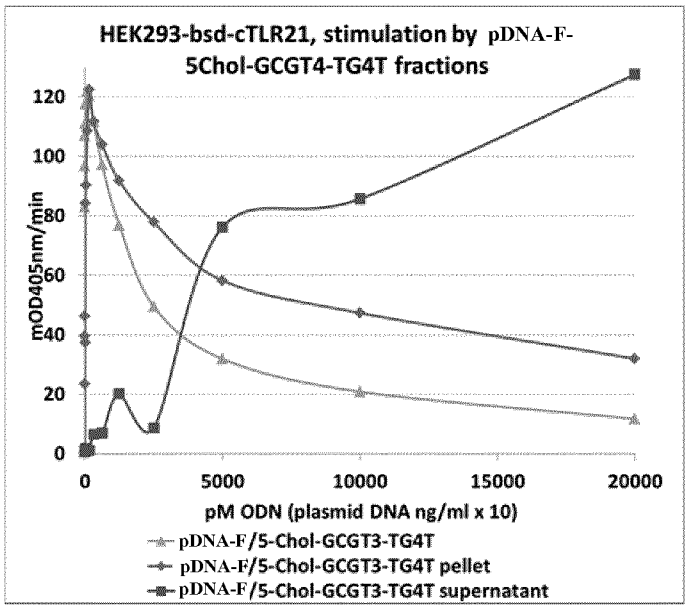

HEK293-bsd-cTLR21, stimulation by pDNA-F-5Chol-GCGT4-TG4T fractions
mOD405nm/min
120
100
80
60
40
20
0
0
5000
10000
15000
20000
pM ODN (plasmid DNA ng/ml x 10)
pDNA-F/5-Chol-GCGT3-TG4T
pDNA-F/5-Chol-GCGT3-TG4T pellet
pDNA-F/5-Chol-GCGT3-TG4T supernatant

FIG. 9A

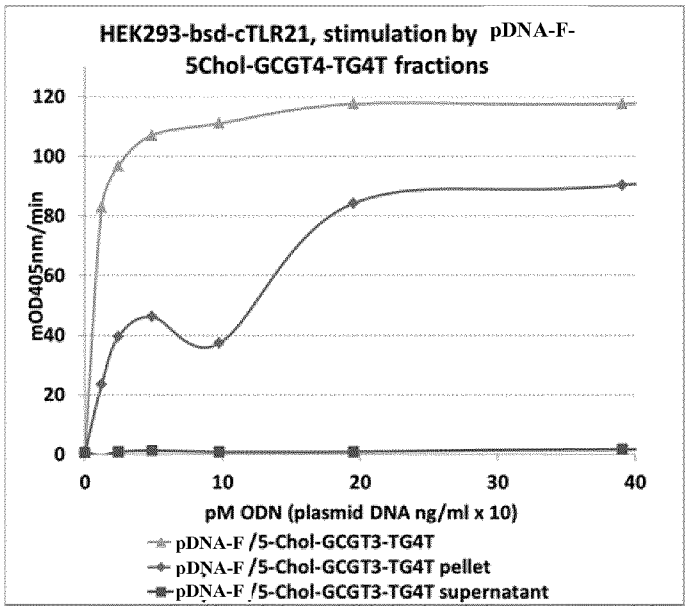

HEK293-bsd-cTLR21, stimulation by pDNA-F-5Chol-GCGT4-TG4T fractions
mOD405nm/min
120
100
80
60
40
20
0
0
10
20
30
40
pM ODN (plasmid DNA ng/ml x 10)
pDNA-F /5-Chol-GCGT3-TG4T
pDNA-F /5-Chol-GCGT3-TG4T pellet
pDNA-F /5-Chol-GCGT3-TG4T supernatant

FIG. 9B

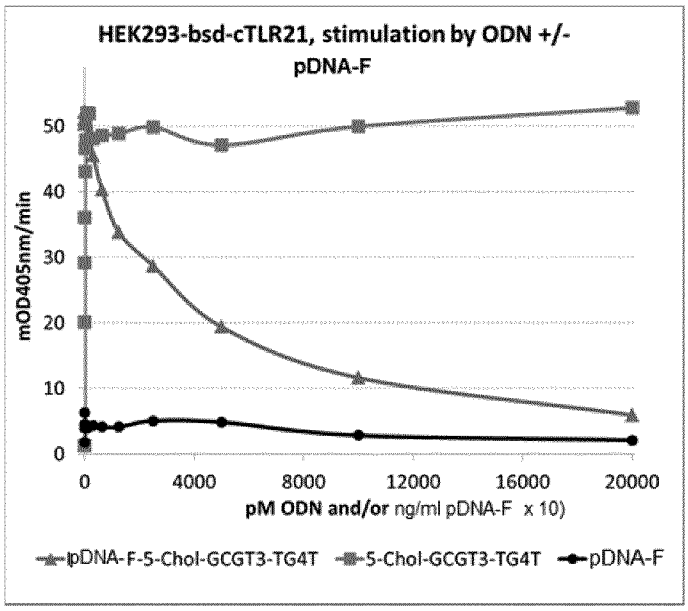


FIG. 10A

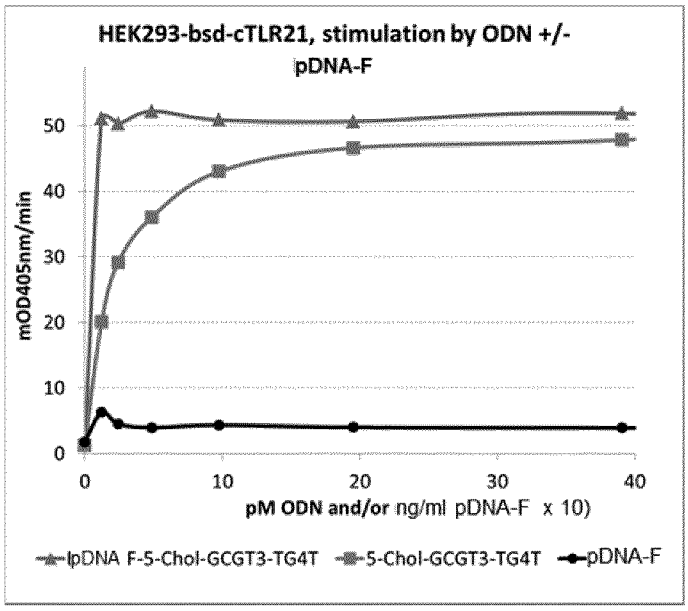


FIG. 10B

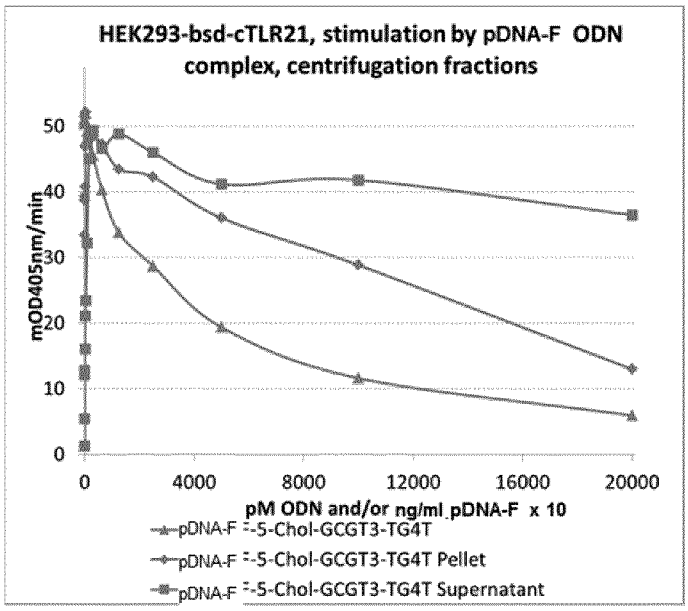


FIG. 11A

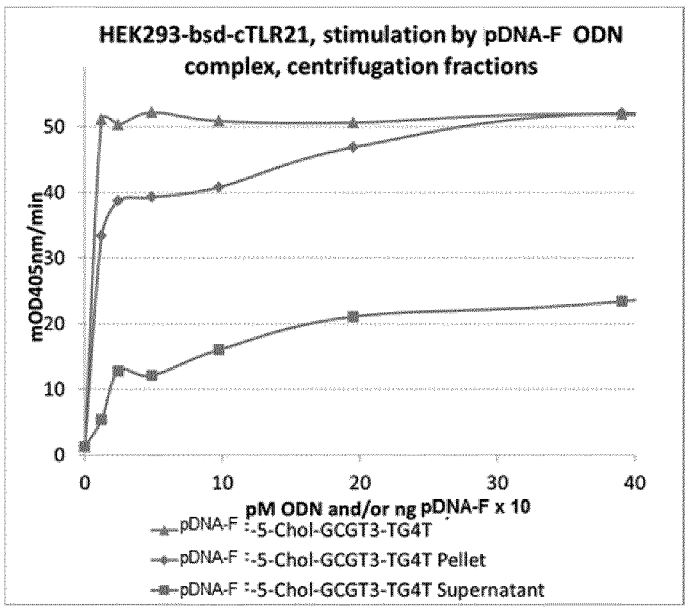


FIG. 11B

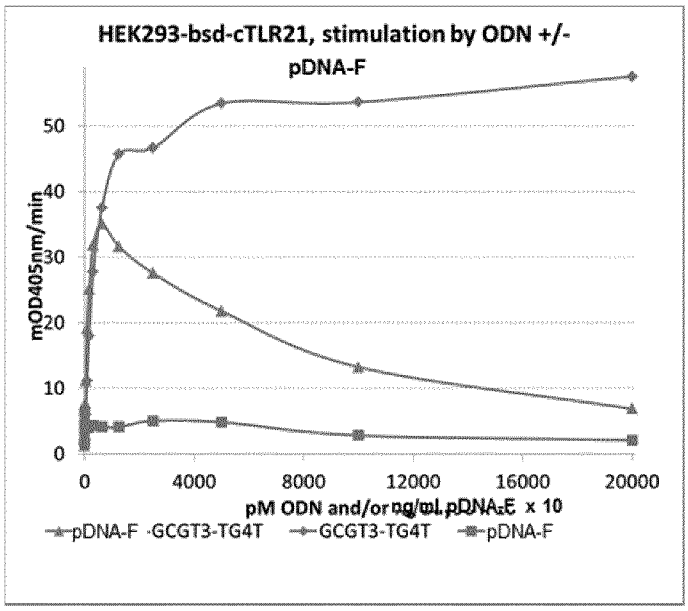


FIG. 12A

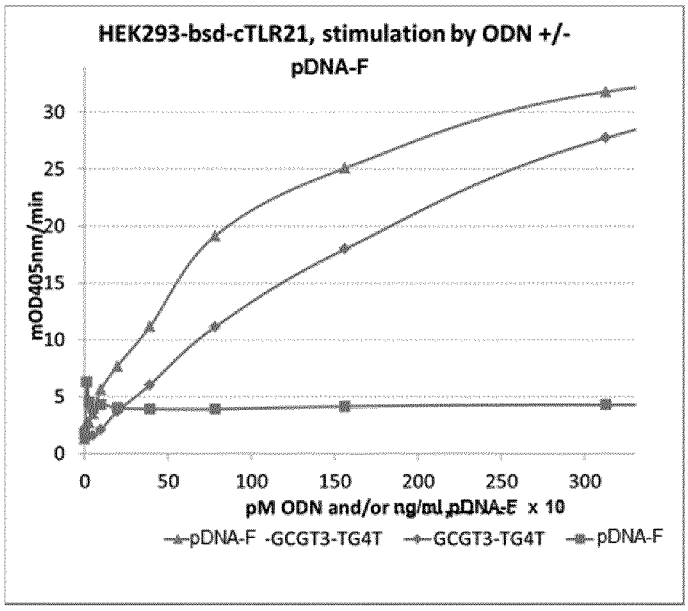


FIG. 12B

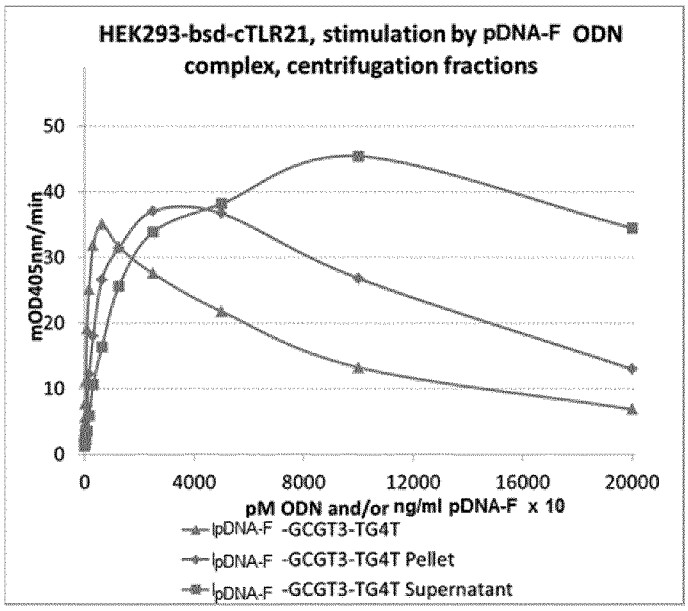


FIG. 13A

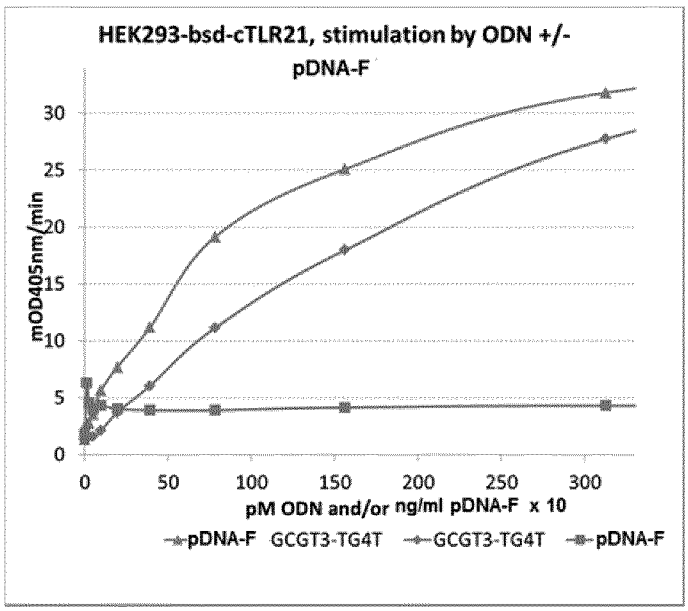


FIG. 13B

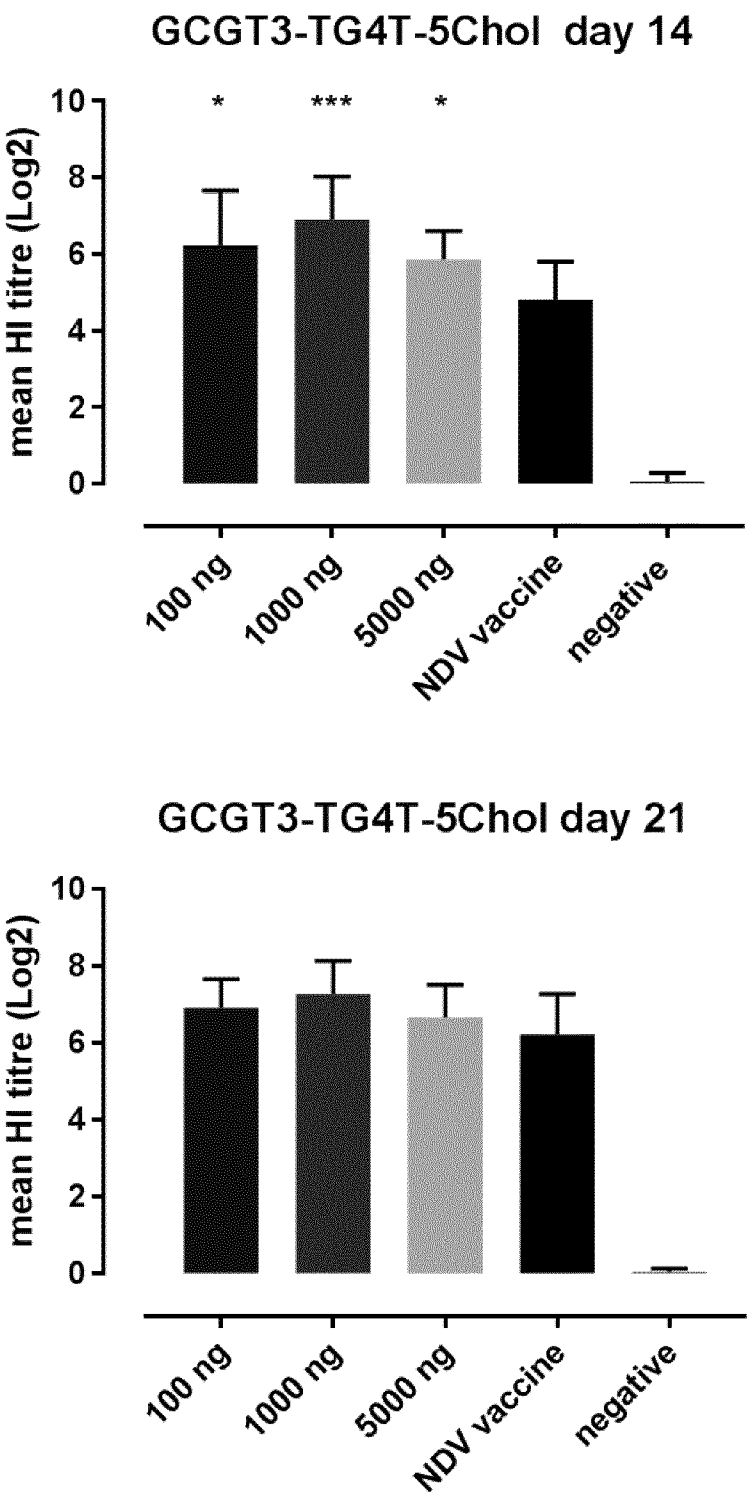

GCGT3-TG4T-5Chol day 14
mean HI titre (Log2)
10
8
6
4
2
0
*
***
*
100 ng
1000 ng
5000 ng
NDV vaccine
negative
GCGT3-TG4T-5Chol day 21
mean HI titre (Log2)
10
8
6
4
2
0
100 ng
1000 ng
5000 ng
NDV vaccine
negative

FIG. 14

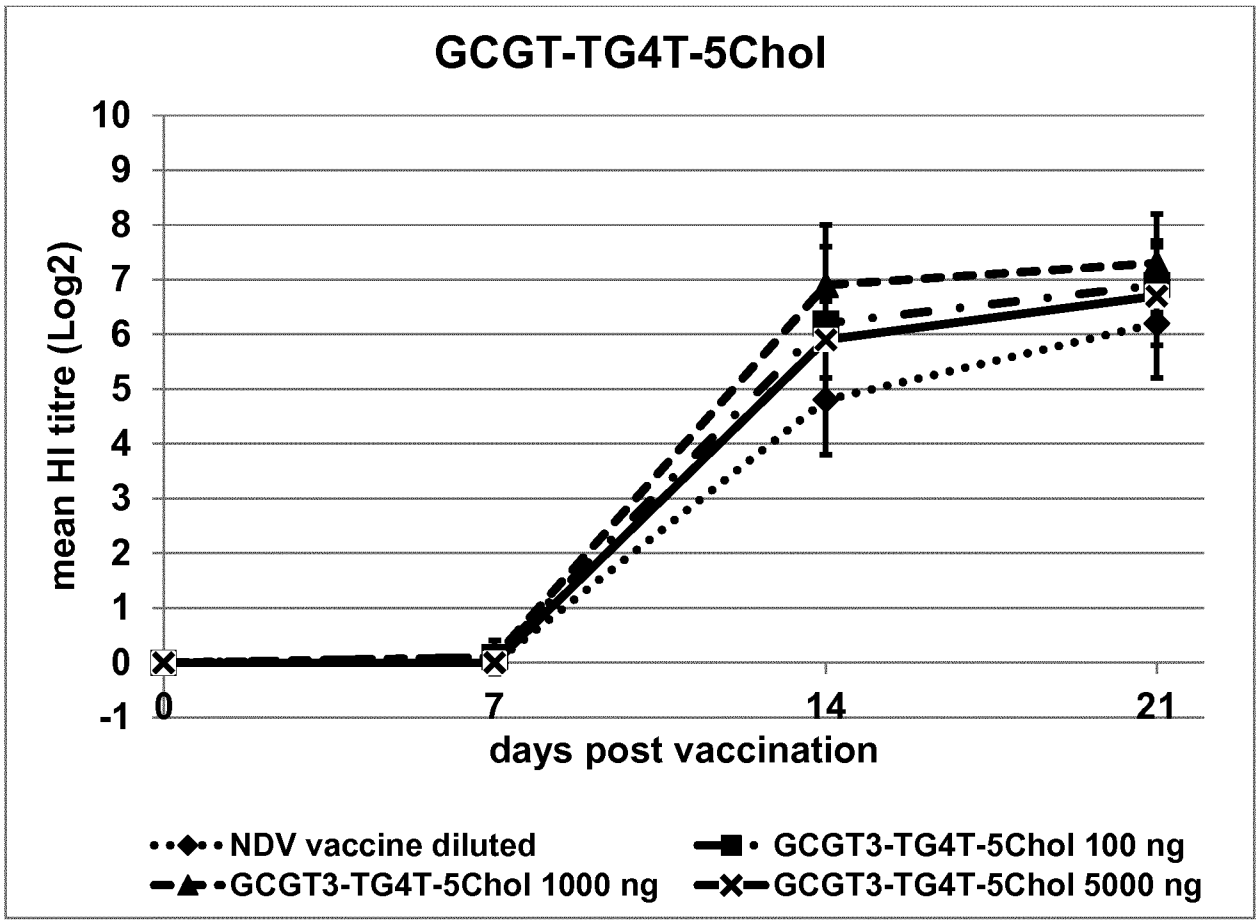

GCGT-TG4T-5Chol
mean HI titre (Log2)
10
9
8
7
6
5
4
3
2
1
0
-1
0
7
14
21
days post vaccination
NDV vaccine diluted
GCGT3-TG4T-5Chol 100 ng
GCGT3-TG4T-5Chol 1000 ng
GCGT3-TG4T-5Chol 5000 ng

FIG. 15

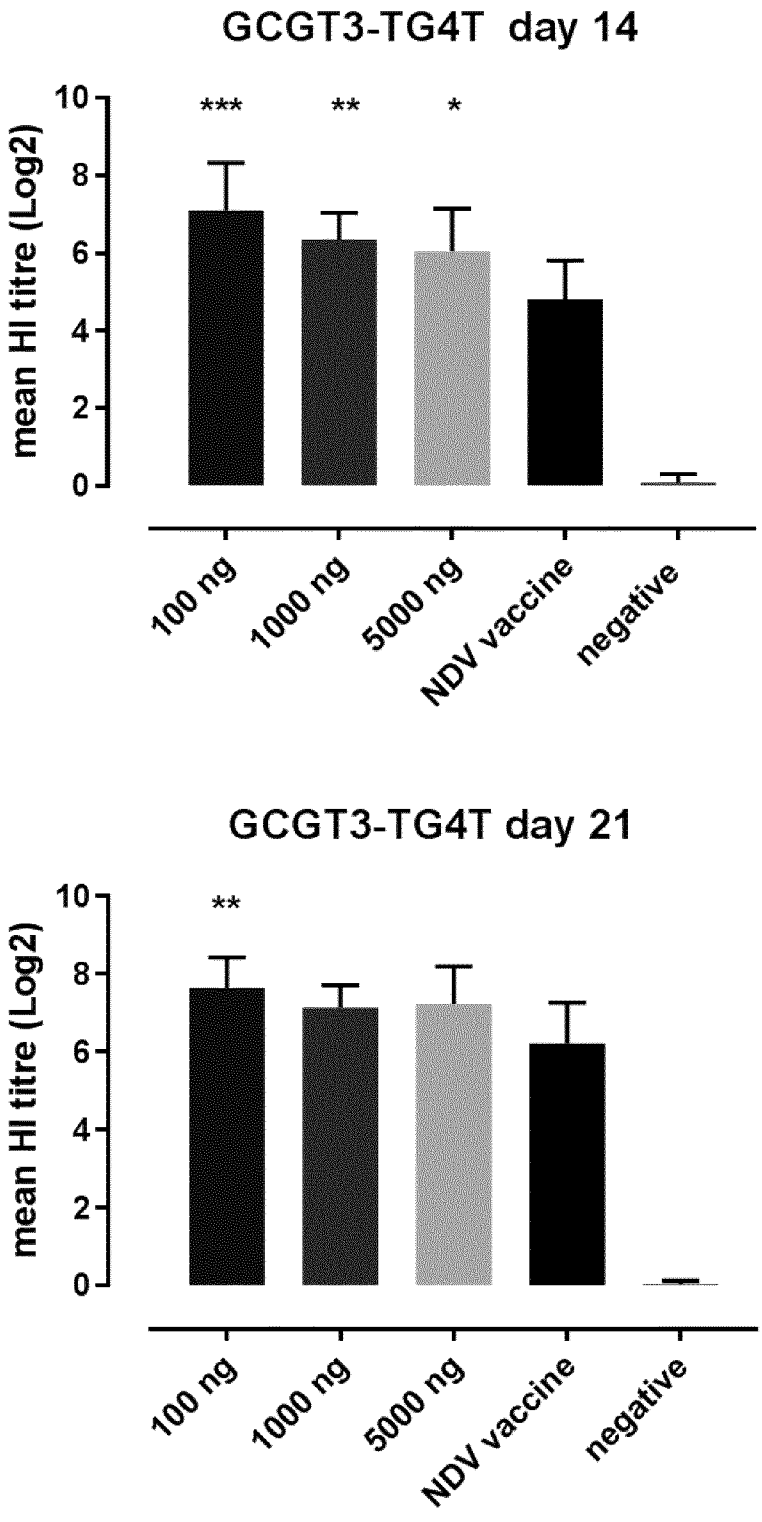

GCGT3-TG4T day 14
mean HI titre (Log2)
10
8
6
4
2
0
***
**
*
100 ng
1000 ng
5000 ng
NDV vaccine
negative
GCGT3-TG4T day 21
mean HI titre (Log2)
10
8
6
4
2
0
**
100 ng
1000 ng
5000 ng
NDV vaccine
negative

FIG. 16

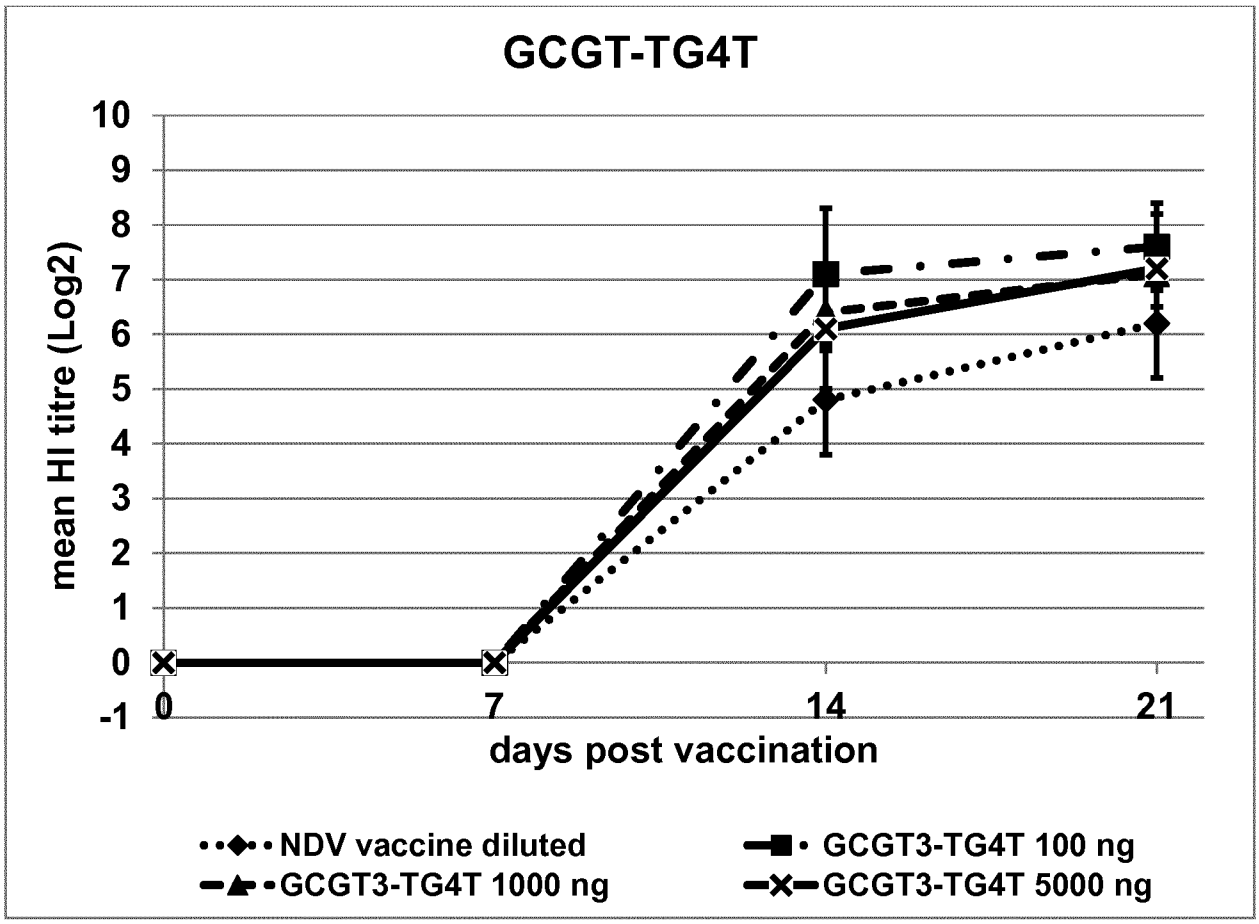

GCGT-TG4T
mean HI titre (Log2)
10
9
8
7
6
5
4
3
2
1
0
-1
0
7
14
21
days post vaccination
NDV vaccine diluted
GCGT3-TG4T 100 ng
GCGT3-TG4T 1000 ng
GCGT3-TG4T 5000 ng

FIG. 17

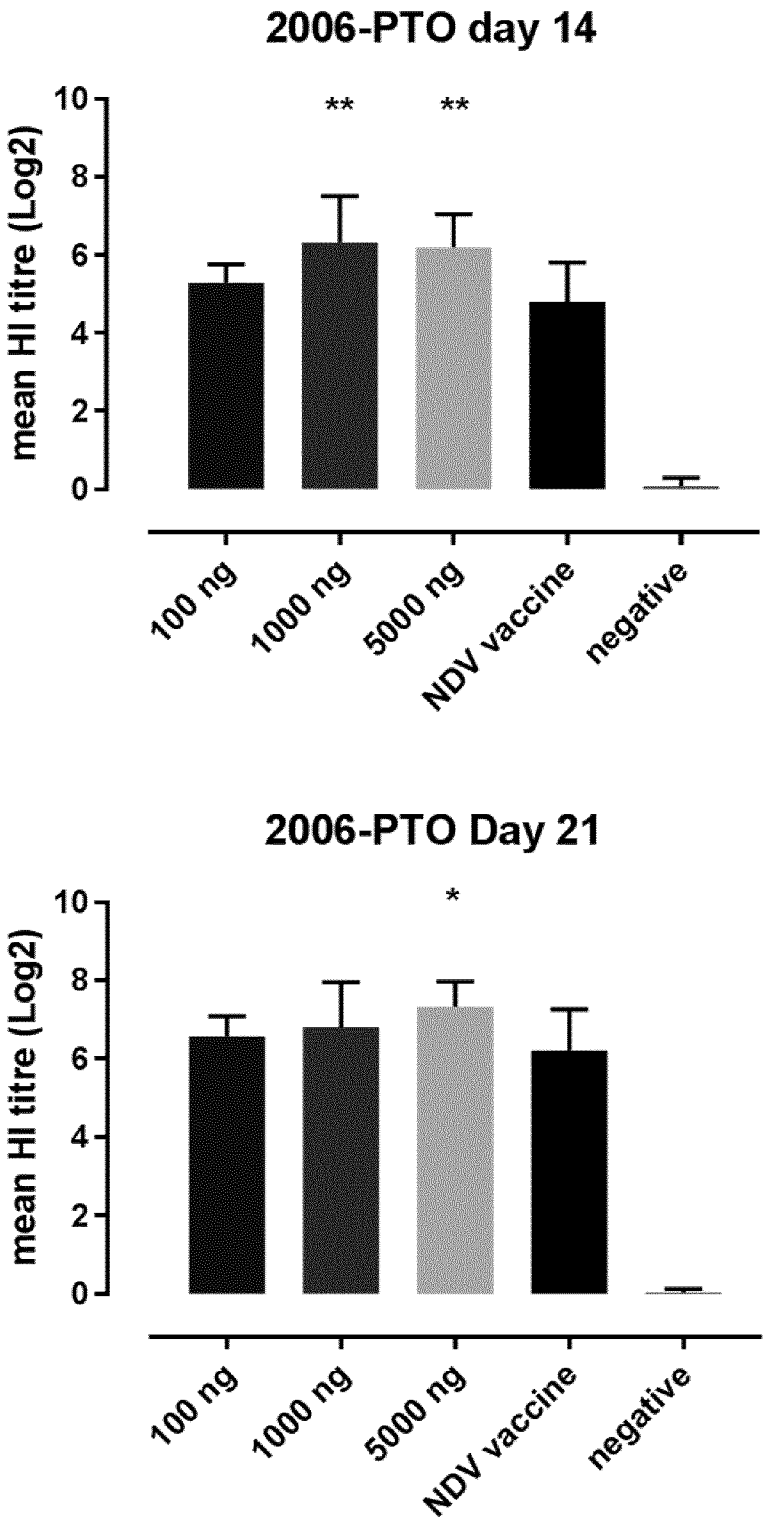

2006-PTO day 14
mean HI titre (Log2)
10
8
6
4
2
0
**
**
100 ng
1000 ng
5000 ng
NDV vaccine
negative
2006-PTO Day 21
mean HI titre (Log2)
10
8
6
4
2
0
*
100 ng
1000 ng
5000 ng
NDV vaccine
negative

FIG. 18

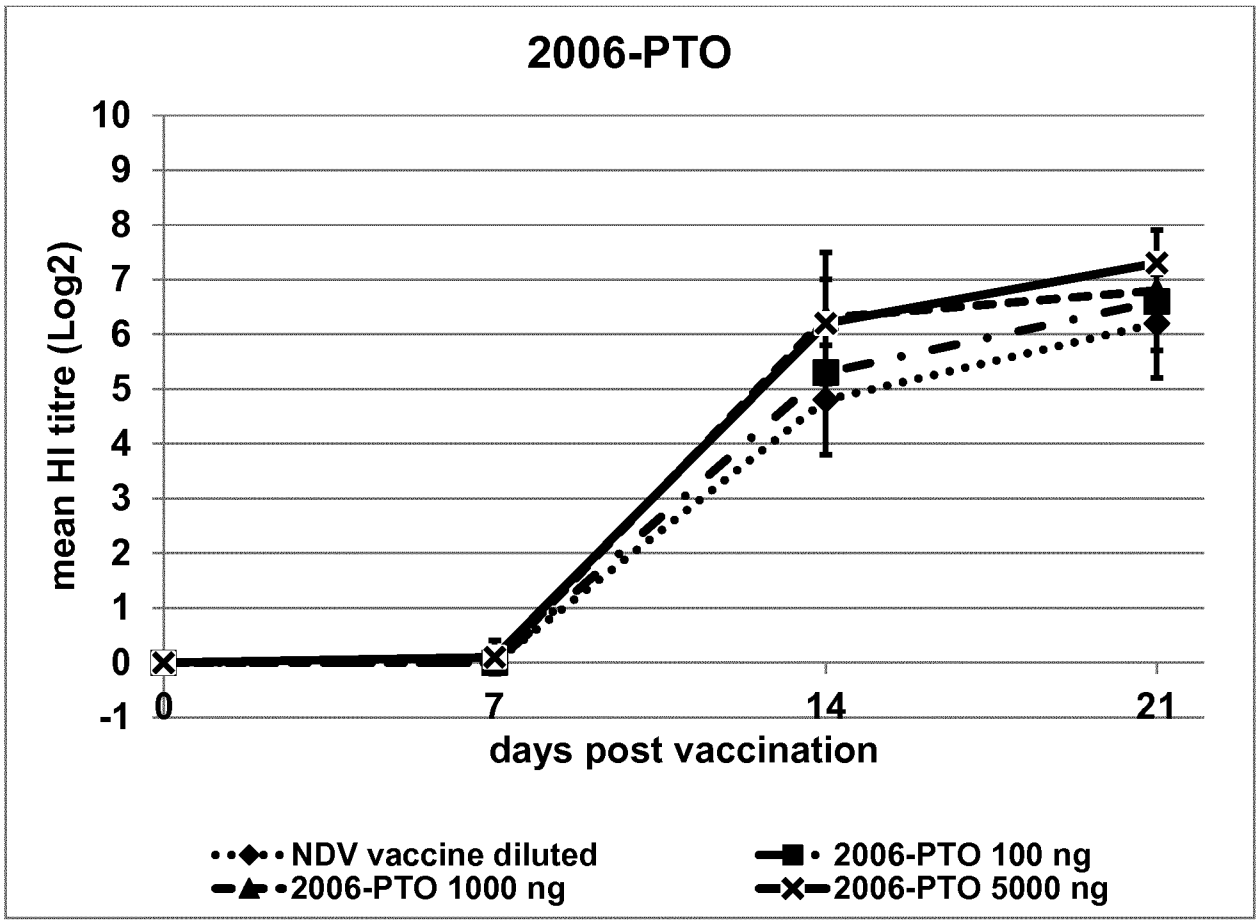

2006-PTO
mean HI titre (Log2)
10
9
8
7
6
5
4
3
2
1
0
-1
0
7
14
21
days post vaccination
NDV vaccine diluted
2006-PTO 100 ng
2006-PTO 1000 ng
2006-PTO 5000 ng

FIG. 19

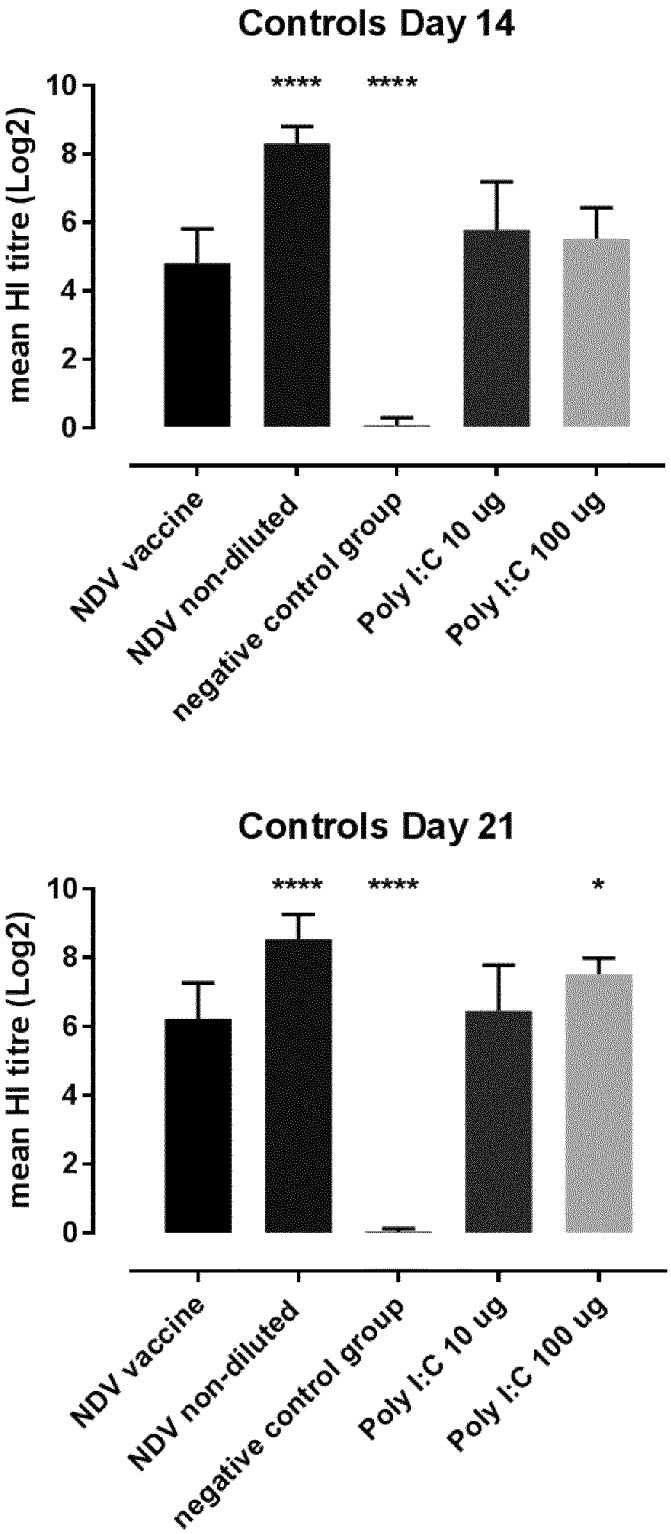

Controls Day 14
mean HI titre (Log2)
10
8
6
4
2
0
****
****
NDV vaccine
NDV non-diluted
negative control group
Poly I:C 10 ug
Poly I:C 100 ug
Controls Day 21
mean HI titre (Log2)
10
8
6
4
2
0
****
****
*
NDV vaccine
NDV non-diluted
negative control group
Poly I:C 10 ug
Poly I:C 100 ug

FIG. 20

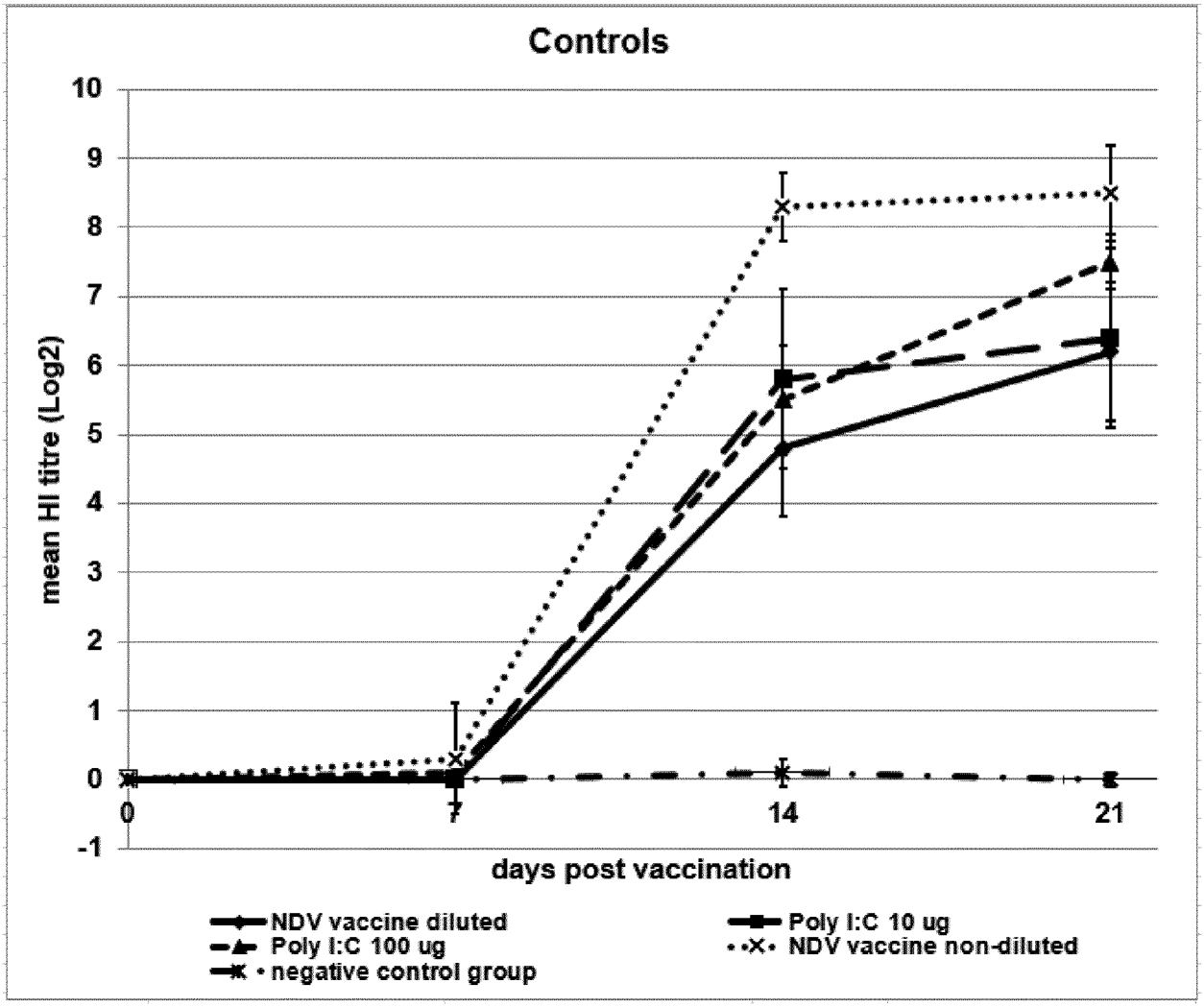

Controls
mean HI titre (Log2)
10
9
8
7
6
5
4
3
2
1
0
-1
0
7
14
21
days post vaccination
NDV vaccine diluted
Poly I:C 10 ug
Poly I:C 100 ug
NDV vaccine non-diluted
negative control group

FIG. 21

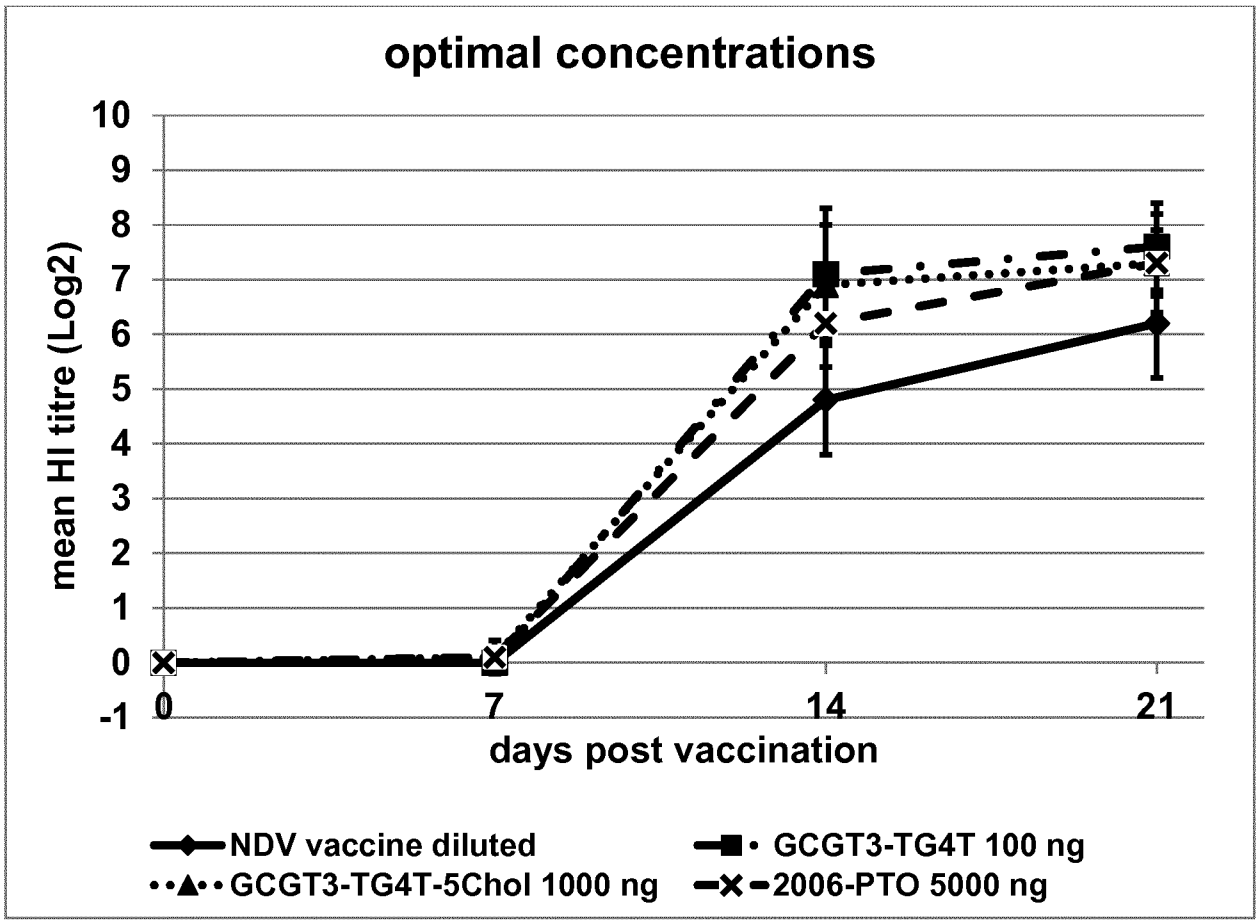

optimal concentrations
mean HI titre (Log2)
10
9
8
7
6
5
4
3
2
1
0
-1
0
7
14
21
days post vaccination
NDV vaccine diluted
GCGT3-TG4T 100 ng
GCGT3-TG4T-5Chol 1000 ng
2006-PTO 5000 ng

FIG. 22

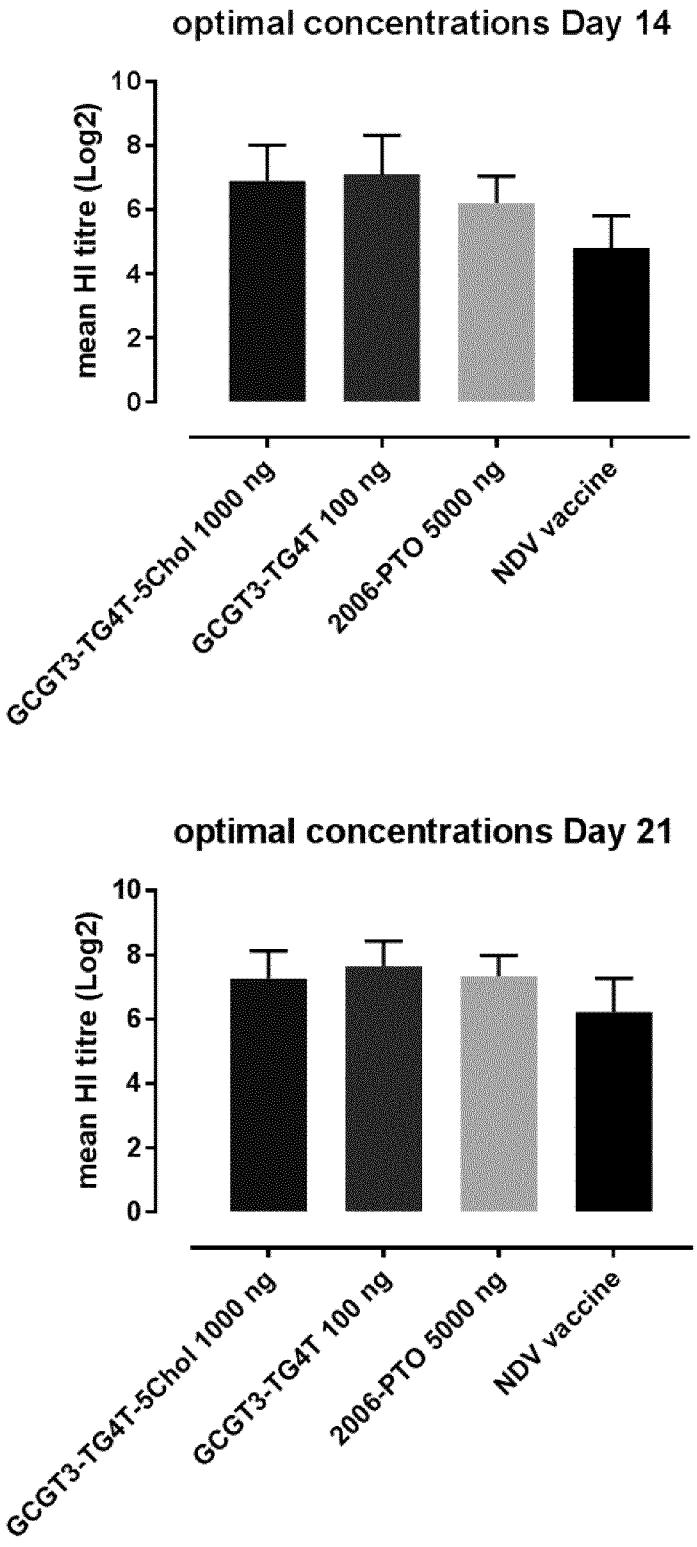

optimal concentrations Day 14
mean HI titre (Log2)
10
8
6
4
2
0
GCGT3-TG4T-5Chol 1000 ng
GCGT3-TG4T 100 ng
2006-PTO 5000 ng
NDV vaccine
optimal concentrations Day 21
mean HI titre (Log2)
10
8
6
4
2
0
GCGT3-TG4T-5Chol 1000 ng
GCGT3-TG4T 100 ng
2006-PTO 5000 ng
NDV vaccine

FIG. 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/083956

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/39 C07H21/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07H

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/084951 A1 (BAYER ANIMAL HEALTH GMBH [DE]; ABRAHAM ALBERT [US]; KEIL DANIEL [US];) 28 June 2012 (2012-06-28) see whole doc. esp. par. 73 and claim 1,	1-47
Y	WO 96/02555 A1 (UNIV IOWA RES FOUND [US]) 1 February 1996 (1996-02-01) see whole doc. esp. claim 5	1-47

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2019	13/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mueller, Frank

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GOMIS SUSANTHA ET AL: "Protection of chickens against Escherichia coli infections by DNA containing CpG motifs", INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 71, no. 2, 1 February 2003 (2003-02-01), pages 857-863, XP002596620, ISSN: 0019-9567, DOI: 10.1128/IAI.71.2.857-863.2003 -----	1-47
A	WO 2004/016805 A2 (COLEY PHARM GROUP INC [US]; COLEY PHARM GMBH [DE]) 26 February 2004 (2004-02-26) see whole doc., sp. claims, example and par. 72, 2. par. -----	1-47

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/083956

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/083956

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