



(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2018/12/07
(87) Date publication PCT/PCT Publication Date: 2019/06/20
(85) Entrée phase nationale/National Entry: 2020/06/12
(86) N° demande PCT/PCT Application No.: EP 2018/083958
(87) N° publication PCT/PCT Publication No.: 2019/115386
(30) Priorités/Priorities: 2017/12/15 (EP17207746.3);
2017/12/15 (EP17207740.6); 2017/12/15 (EP17207750.5)

(51) Cl.Int./Int.Cl. *C12N 15/117* (2010.01),
A61K 31/7088 (2006.01), *A61P 37/04* (2006.01),
C12N 15/85 (2006.01)
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(54) Titre : OLIGONUCLEOTIDES IMMUNOSTIMULANTS
(54) Title: IMMUNOSTIMULATORY OLIGONUCLEOTIDES

(57) Abrégé/Abstract:

Disclosed herein are immunostimulatory oligonucleotides and compositions and methods of use thereof. More specifically, immunostimulatory oligonucleotides, methods of optimizing the immunostimulatory properties of oligonucleotides, and methods of using the immunostimulatory oligonucleotides to elicit a toll-like receptor 21 (TLR21)-mediated immune response are disclosed.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property

Organization

International Bureau

(43) International Publication Date

20 June 2019 (20.06.2019)



(10) International Publication Number

WO 2019/115386 A1

(51) International Patent Classification:

C12N 15/117 (2010.01)

(21) International Application Number:

PCT/EP2018/083958

(22) International Filing Date:

07 December 2018 (07.12.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

17207750.5 15 December 2017 (15.12.2017) EP

17207740.6 15 December 2017 (15.12.2017) EP

17207746.3 15 December 2017 (15.12.2017) EP

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

(54) Title: IMMUNOSTIMULATORY OLIGONUCLEOTIDES

(57) Abstract: Disclosed herein are immunostimulatory oligonucleotides and compositions and methods of use thereof. More specifically, immunostimulatory oligonucleotides, methods of optimizing the immunostimulatory properties of oligonucleotides, and methods of using the immunostimulatory oligonucleotides to elicit a toll-like receptor 21 (TLR21)-mediated immune response are disclosed.



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IMMUNOSTIMULATORY OLIGONUCLEOTIDES

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_168028_SL.txt and is 78,795 bytes in size.

FIELD OF THE INVENTION

[0003] Disclosed herein are immunostimulatory oligonucleotides and compositions and methods of use thereof. More specifically, immunostimulatory oligonucleotides, methods of optimizing the immunostimulatory properties of oligonucleotides, and methods of using the immunostimulatory oligonucleotides to elicit a toll-like receptor 21 (TLR21)-mediated immune response are disclosed.

BACKGROUND OF THE INVENTION

[0004] Some molecular attributes of microorganisms including proteins and other antigens on a microbe's surface, as well as internal compositions such as certain motifs contained within a microbe's genome (e.g., unmethylated CpG motifs), can be recognized by a host organism's immune system and elicit immune responses. Interaction between these molecular attributes, or pathogen associated molecular patterns (PAMPs), and a host's cognate pathogen recognition receptors can initiate cell signaling cascades involved in immune responses. Toll-like receptor 21 (TLR21) is the chicken functional homolog of mammalian toll-like receptor 9 (TLR9) and a PAMP receptor capable of recognizing unmethylated CpG motifs. Activation of TLR21 by nucleic acids having these CpG motifs has been shown to activate cellular signals involved in immune responses to microbial infection.

[0005] Comprehension of TLR21's role in the immune response in chickens has not led to a shift in disease prevention or treatment in the poultry industry. Large populations of poultry housed in brooder facilities are at increased risk of microbial infections at all stages of life due to inherently crowded and nonsterile environments, but currently available prophylactic compositions and post-infection treatments generally do not elicit PAMP-mediated immune responses. Instead, large scale production facilities rely on commercially available vaccines and antibiotics to prevent or curtail infectious outbreaks. Although antibiotics are becoming disfavored due to concerns of resistance and unintended consequences of consuming treated meat, antibiotic administration remains a standard operating procedure in many agricultural settings, including large-scale brooder houses, and adoption of new methods can be prohibitively expensive and burdensome. One hindrance to adopting TLR21 agonists as a prophylactic measure or as a treatment for infection includes the inefficiencies associated with screening large numbers of candidate compounds, which effectively disincentivizes research to identify such agonists.

[0006] Thus, there is a need for TLR21 stimulatory compositions, methods for identifying them, and optimizing the immunostimulatory properties of the compositions. The disclosed methods and compositions are directed to these and other important needs.

SUMMARY OF THE INVENTION

[0007] Disclosed herein are oligonucleotides comprising at least one CpG motif and a guanine nucleotide enriched ("guanine-enriched") sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

[0008] Also disclosed herein are oligonucleotides comprising a 5'-cholesteryl modification with at least one CpG motif and with or without a guanine nucleotide enriched sequence within four nucleotides of the 5' terminus of the oligonucleotide.

[0009] Also provided are methods of stimulating toll-like receptor 21 (TLR21) comprising administering to a subject in need thereof an immunostimulatory oligonucleotide having at least one CpG motif and an guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

[0010] Methods for increasing TLR21-stimulatory activity of an oligonucleotide having at least one CpG motif comprising fusing the 5' end of the oligonucleotide to a guanine nucleotide enriched sequence are also disclosed.

[0011] Provided herein are methods for eliciting an immune response in a subject comprising administering to a subject an oligonucleotide having at least one CpG dinucleotide motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide to the subject.

BRIEF DESCRIPTIONS 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** is a plasmid map of pcDNATM3.1(+).

[0014] **FIG. 2** compares the dose response curves of TNF- α -stimulated HEK293-NF κ B cells and HEK293-NF κ B-bsd-cTLR21.

[0015] **FIG. 3A and FIG. 3B** graphically depict the stimulatory effects of 2006-PTO and 2006-PDE on HEK293-bsd and HEK293-bsd-cTLR21 cells.

[0016] **FIG. 4A and FIG. 4B** graphically depict the stimulatory effects of increasing numbers of guanine residues at the 3' terminus of the 2006-PDE oligonucleotide.

[0017] **FIG. 5A and FIG. 5B** graphically depict the stimulatory effects of increasing numbers of guanine residues at the 5' terminus of the 2006-PDE oligonucleotide.

[0018] **FIG. 6** illustrates the negative logarithm (log₁₀) of the half maximum effective concentration (pEC₅₀) of 2006-PDE oligonucleotides having increasing numbers of guanine residues at their 3' or 5' termini.

[0019] **FIG. 7A and FIG. 7B** illustrate aggregation of the 2006-PDE oligonucleotides having increasing numbers of guanines at their 5' and 3' termini, respectively.

[0020] **FIG. 8A and FIG. 8B** graphically depict the stimulatory effects of 2006-PDE oligonucleotide with six consecutive guanine (5dG6), adenine (5dA6), cytosine (5dC6), or thymine (5dT6) residues at its 5' terminus.

[0021] **FIG. 9A and FIG. 9B** graphically depict the effect of disruption of the CpG motif(s) on the stimulation of TLR21 by oligonucleotides.

[0022] **FIG. 10A and FIG. 10B** graphically depict the stimulatory effects of guanine runs on the 3' and 5' end, respectively, of oligonucleotides having phosphodiester or phosphorothioate backbones.

[0023] **FIG. 11A and FIG. 11B** illustrate the effects on TLR21 stimulation of a single adenine substitution within the six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs, while **FIG. 11C and FIG. 11D** illustrate the effects on TLR21 stimulation of a two adenine substitution within the six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs.

[0024] **FIG. 12A and FIG. 12B** illustrate the effects on TLR21 stimulation of a single cytosine substitution within the six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs, while **FIG. 12C and FIG. 12D** illustrate the effects on TLR21 stimulation of a two cytosine substitution within the six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs.

[0025] **FIG. 13A and FIG. 13B** illustrate the effects on TLR21 stimulation of a single thymine substitution within the six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs, while **FIG. 13C and FIG. 13D** illustrate the effects on TLR21 stimulation of a two thymine substitution within the six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs.

[0026] **FIG. 14** demonstrates the positional effect on TLR21 stimulation of single nucleotide substitutions in a six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs (SEQ ID NO: 272).

[0027] **FIG. 15** demonstrates the positional effect on TLR21 stimulation of double nucleotide substitutions in a six guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs (SEQ ID NO: 272).

[0028] **FIG. 16A and FIG. 16B** illustrate the effects on TLR21 stimulation of a single adenine substitution within a four guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs.

[0029] **FIG. 17A and FIG. 17B** illustrate the effects on TLR21 stimulation of a single cytosine substitution within a four guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs.

[0030] **FIG. 18A and FIG. 18B** illustrate the effects on TLR21 stimulation of a single thymine substitution within a four guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs.

[0031] **FIG. 19** demonstrates the positional effect on TLR21 stimulation of single nucleotide substitutions in a four guanine run on the 5' terminus of an oligonucleotide having multiple CpG motifs (SEQ ID NO: 273).

[0032] **FIGs. 20A – 20K** illustrate the effects of fusing five guanine run on the 3' terminus, a four guanine run on the five prime terminus, and a six guanine run on the 5' terminus of CpG-containing oligodeoxynucleotide sequences implicated in the literature. **FIG. 20A** graphically illustrates the TLR21 stimulatory activity of ODNs 1668, 1668-3dG5, 1668-5dG4, and 1668-5dG6. **FIG. 20B** graphically illustrates the TLR21 stimulatory activity of ODN 1826-3dG5, 1826-5dG4, and 1826-5dG6. **FIG. 20C** graphically illustrates the TLR21 stimulatory activity of ODNs BW006, BW006-3dG5, BW006-5dG4, and BW006-5dG6. **FIG. 20D** graphically illustrates the TLR21 stimulatory activity of ODNs D-SLO1, D-SLO1-3dG5, D-SLO1-5dG4, and D-SLO1-5dG6. **FIG. 20E** graphically illustrates the TLR21 stimulatory activity of ODNs M362, M362-3dG5, M362-5dG4, and M362-5dG6. **FIG. 20F** graphically illustrates the TLR21 stimulatory activity of ODNs 2395, 2395-5dG4, and 2395-5dG6. **FIG. 20G** graphically illustrates the TLR21 stimulatory activity of ODNs 2007-PDE, 2007-PDE -3dG5, 2007-PDE -5dG4, and 2007-PDE -5dG6. **FIG. 20H** graphically illustrates the TLR21 stimulatory activity of ODNs CPG-685 and CPG-685-5dG6. **FIG. 20I** graphically illustrates the TLR21 stimulatory activity of ODNs CPG-202 and CPG-202-5dG6. **FIG. 20J** graphically illustrates the TLR21 stimulatory activity of ODNs CPG-2000 and CPG-2000-5dG6. **FIG. 20K** graphically illustrates the TLR21 stimulatory activity of ODNs CPG-2002 and CPG-2002-5dG6.

[0033] **FIG. 21A** graphically depicts the impact of fusing known telomeric sequences to 2006-PDE and 2006-PDE-T4; **FIG. 21B and FIG. 21C** graphically depict the impact of fusing telomeric or promoter sequences to 2006-PDE-T4.

[0034] **FIG. 22A and FIG. 22B** illustrate the impact of fusing known telomeric sequences to 2006-PDE.

[0035] **FIG. 23A and FIG. 23B** show base-pairing arrangement of a tetramer of oligonucleotides having G-quartet sequences and the orientation of the oligonucleotides comprising the tetramer, respectively ("TGGGGT" disclosed as SEQ ID NO: 265); **FIG. 23C** illustrates the

interactions of oligonucleotides when forming a G-quartet or a G-wire conformation (SEQ ID NO: 257); **FIG. 23D** is an image of a G-wire conformation (SEQ ID NO: 257).

[0036] **FIG. 24A and FIG. 24B** depict the effect on TLR21 stimulation by adding guanine nucleotide enriched sequences to the 5' end of an oligonucleotide having CpG motifs.

[0037] **FIG. 25** depicts the presence of aggregated oligodeoxynucleotides having a G-wire sequence.

[0038] **FIG. 26A, FIG. 26B, and FIG. 26C** graphically depict the stimulatory impact of the nucleotides immediately adjacent to a CpGpA motif. **FIG. 26A** depicts the TLR21 stimulatory activity the basal oligonucleotides. **FIG. 26B** depicts the same oligonucleotides with an additional 5' dG₆ sequence. **FIG. 26C** depicts the basal oligonucleotides with an additional 5' Gwire2 sequence.

[0039] **FIG. 27A, FIG. 27B, and FIG. 27C** graphically depict the stimulatory impact of the nucleotides immediately adjacent to a CpGpG motif. **FIG. 27A** depicts the TLR21 stimulatory activity the basal oligonucleotides. **FIG. 27B** depicts the same oligonucleotides with an additional 5' dG₆ sequence. **FIG. 27C** depicts the same oligonucleotides with an additional 5' Gwire2 sequence.

[0040] **FIG. 28A, FIG. 28B, and FIG. 28C** graphically depict the stimulatory impact of the nucleotides immediately adjacent to a CpGpC motif. **FIG. 28A** depicts the TLR21 stimulatory activity the basal oligonucleotides. **FIG. 28B** depicts the same oligonucleotides with an additional 5' dG₆ sequence. **FIG. 28C** depicts the same oligonucleotides with an additional 5' Gwire2 sequence.

[0041] **FIG. 29A, FIG. 29B, FIG. 29C, and FIG. 29D** graphically depict the stimulatory impact of the nucleotides immediately adjacent to a CpGpT motif. **FIG. 29A** depicts the TLR21 stimulatory activity the basal oligonucleotides. **FIG. 29B** depicts the same oligonucleotides with an additional 5' dG₆ sequence. **FIG. 29C and FIG. 29D** depict the same oligonucleotides with an additional 5' Gwire2 sequence.

[0042] **FIG. 30** illustrates the stimulatory impact of disrupting the only CpG motif in an oligonucleotide having a 5' Gwire sequence.

[0043] **FIG. 31** illustrates the stimulatory impact of the distance between a 5' Gwire sequence and a CpG motif.

[0044] **FIG. 32** illustrates the stimulatory impact of modifying the number of thymidine 5'-monophosphate nucleotides at the 3' end of an oligonucleotide having a 5' Gwire2 sequence and a CpG motif.

[0045] **FIG. 33A and FIG. 33B** compare the immunostimulatory properties of oligonucleotides having different 5' G-wire sequences sequence.

[0046] **FIG. 34** depicts the structure-activity of an oligonucleotide having a 5' Gwire2 sequence. Figure discloses SEQ ID NOS 189, 269, and 270, respectively, in order of appearance.

[0047] **FIG. 35A and FIG. 35B** compare the immunostimulatory capabilities of oligonucleotides having a single, double or triple GCGT sequence element near the 3' of an oligonucleotide with a 5' Gwire2 sequence and CpG motifs.

[0048] **FIG. 36A and FIG. 36B** compare the immunostimulatory capabilities of oligonucleotides having a single, double or triple GCGA sequence element near the 3' of an oligonucleotide with a 5' Gwire2 sequence and CpG motifs.

[0049] **FIG. 37A and FIG. 37B** compare the immunostimulatory capabilities of oligonucleotides having a single, double or triple ACGC sequence element near the 3' of an oligonucleotide with a 5' Gwire2 sequence and CpG motifs.

[0050] **FIG. 38A and FIG. 38B** compare the immunostimulatory capabilities of oligonucleotides having a single, double or triple TCGC sequence element near the 3' of an oligonucleotide with a 5' Gwire2 sequence and CpG motifs.

[0051] **FIG. 39A and FIG. 39B** compare the immunostimulatory capabilities of oligonucleotides having a single, double or triple CCGC sequence element near the 3' of an oligonucleotide with a 5' Gwire2 sequence and CpG motifs.

[0052] **FIG. 40** compares the immunostimulatory capabilities of oligonucleotides having a single, double or triple GCGG sequence element near the 3' of an oligonucleotide with a Gwire2 sequence and CpG motifs.

[0053] **FIG. 41A and FIG. 41B** compare the immunostimulatory effects of inserting one to four thymine nucleotides between two CpG motifs in an oligonucleotide.

[0054] **FIG. 42A and FIG. 42B** illustrate the stimulatory effect of a single nucleotide separation between two CpG motifs or an abasic spacer between the two CpG motifs.

[0055] **FIG. 43A to 43E** depicts different structural bridges between CpG elements in an oligonucleotide. FIG. 43A depicts the structure of a T1 spacer. FIG. 43B depicts the structure T3

spacer. FIG. 43C depicts the structure of an abasic spacer. FIG. 43D depicts the structure of 1,3-propanediol spacer. FIG. 43E depicts the structure of a hexaethyleneglycol spacer.

[0056] **FIG. 44A and FIG. 44B** show the stimulatory impact of inserting a C3 and a C18 spacer between CpG motifs.

[0057] **FIG. 45A and FIG. 45B** depict the immunostimulatory impact of increasing numbers of CpG motifs in an oligonucleotide comprising a 5'-Gwire2 motif, the CpG motifs being separated by a C3 spacer.

[0058] **FIG. 46A and FIG. 46B** graphically illustrate the immunostimulation of TLR21 by oligonucleotides with a TGGGGT-sequence (SEQ ID NO: 265) at the 5' end and between one and five CpG motifs, each separated by C3 spacers.

[0059] **FIG. 47** depicts abasic diol-based spacers.

[0060] **FIG. 48** depicts a C8 spacer, a basal spacer, and an abasic deoxyribose bridge spacer.

[0061] **FIG. 49A and FIG. 49B** graphically display TLR21 stimulation by oligonucleotides having a GGGGTTGGGG (SEQ ID NO: 257) 5' terminal sequences and CpG motifs, and wherein the CpG motifs are separated by propanediol or an abasic deoxyribose bridge.

[0062] **FIG. 50A and FIG. 50B** illustrate the TLR21 stimulation capabilities of oligonucleotides having CpG motifs and a G-wire sequence, wherein the CpG motifs are separated by ethanediol, propanediol, butanediol, pentanediol, and hexanediol.

[0063] **FIG. 51** illustrates the impact of different diol-based spacers between CpG elements on the stimulation of TLR21.

[0064] **FIG. 52A and FIG. 52B** depict TLR21 stimulation after exposure to oligonucleotides having either ACGC or CCGC CpG sequence elements separated by propanediol or hexaethylene glycol and a G-wire 5' terminal sequence.

[0065] **FIG. 53A and FIG. 53B** depict TLR21 stimulation after exposure to oligonucleotides having either ACGC or CCGC CpG sequence elements separated by propanediol and a TGGGGT (SEQ ID NO: 265) 5' terminal sequence.

[0066] **FIG. 54A and FIG. 54B** depict TLR21 stimulation after exposure to oligonucleotides having a G-wire 5' terminal sequence and CpG motifs separated by either propanediol or hexaethyleneglycol.

[0067] **FIG. 55** illustrates the chemical structure of a cholesterol moiety connected to the 3' deoxyribose moiety by a hexanediol linker.

[0068] **FIG. 56A and FIG. 56B** compare the TLR21 stimulation from an oligonucleotide having multiple CpG motifs, and a 5' Gwire2 sequence to that from the same oligonucleotide having a 3' cholesteryl group.

[0069] **FIG. 57A and FIG. 57B** compare two oligonucleotides having a TGGGGT (SEQ ID NO: 265) 5' end terminal sequence, multiple CpG motifs, and a 3' cholesteryl group.

[0070] **FIG. 58A and FIG. 58B** depict 5' cholesterol modifications to two different deoxynucleotides.

[0071] **FIG. 59A and FIG. 59B** illustrate TLR21 stimulation caused by oligonucleotides having a TGGGGT-5' terminal sequence (SEQ ID NO: 265), multiple CpG motifs, and with or without a 5' cholesterol modification.

[0072] **FIG. 60A and FIG. 60B** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications, wherein the cholesterol derivative is obtained from a different supplier (Sigma Aldrich).

[0073] **FIG. 61A and FIG. 61B** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0074] **FIG. 62A and FIG. 62B** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0075] **FIG. 63A and FIG. 63B** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0076] **FIG. 64** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0077] **FIG. 65A and FIG. 65B** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0078] **FIG. 66A and FIG. 66B** illustrate TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0079] **FIG. 67** illustrates TLR21 stimulation by oligonucleotides with or without 5' cholesterol modifications.

[0080] **FIG. 68A and FIG. 68B** graphically depict the immunostimulatory effects of oligonucleotides modified with increasing numbers of guanine nucleotides on the oligonucleotide's 5' terminus in mouse and human cells, respectively.

[0081] **FIG. 69A and FIG. 69B** graphically depict the immunostimulatory effects of oligonucleotides modified with increasing numbers of guanine nucleotides on the oligonucleotide's 3' terminus in mouse and human cells, respectively.

[0082] **FIG. 70** 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).

[0083] **FIG. 71** depicts mean HI titres (Log2) (with standard deviation) results for ODN1 (GCGT3-TG4T-5Chol) during the entire study.

[0084] **FIG. 72** 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).

[0085] **FIG. 73** depicts mean HI titres (Log2) (with standard deviation) results for ODN2 (GCGT3-TG4T) during the entire study.

[0086] **FIG. 74** 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).

[0087] **FIG. 75** depicts mean HI titres (Log2) (with standard deviation) results for ODN3 (2006-PTO) during the entire study.

[0088] **FIG. 76** 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).

[0089] **FIG. 77** depicts mean HI titres (Log2) (with standard deviation) results for positive and negative control Test Articles during the entire study.

[0090] **FIG. 78** 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.

[0091] **FIG. 79** 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

[0092] 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.

[0093] 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.

[0094] 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.

[0095] 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.

[0096] 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.

[0097] As used herein, the singular forms “a,” “an,” and “the” include the plural.

[0098] As used herein, “fuse” or “fusing” refers to creating a chemical bond between to chemical 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.

[0099] 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 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 (SEQ ID NO: 265).

[0100] 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 nucleotides). 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).

[0101] As used herein, the terms “guanine nucleotide enriched sequence,” “guanine-enriched sequence,” and the like, refer to nucleic acid 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 nucleotide 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.

[0102] As used herein, “inserting” refers to adding specific nucleotide(s) at specific positions during the synthesis of an oligonucleotide.

[0103] As used herein, “parallel orientation” refers to the directional interaction between different oligonucleotides. For example, the circled illustration in FIG. 23B demonstrates four oligonucleotides having parallel orientation, as the tetramer of oligonucleotides are positioned parallel to each other. In some aspects, the individual oligonucleotides can be oriented in the same 5' to 3' direction.

[0104] The term “subject” as used herein is intended to mean any animal, including any type of avian, mammalian, or aquatic species, and in particular chickens. Subjects can be treated using the disclosed methods and with the disclosed compositions.

[0105] The term “TLR21 testing,” or variations thereof, refers to administering oligonucleotides to the HEK293-NFκB-bsd-cTLR21 cell line described in Example 2 to determine if the oligonucleotide stimulates TLR21.

[0106] 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.

[0107] Disclosed herein are recombinant HEK293 cell lines comprising a blasticidin resistance gene and a synthetic SEAP reporter gene construct (“NFκB-SEAP”) as well as a stable cell line co-transfected with the NFκB-SEAP construct and a chicken TLR21 construct (HEK293-NFκB-bsd-cTLR21). This latter cell line can be employed to test the TLR21-mediated immunostimulatory properties of candidate compounds. As demonstrated in the examples, the HEK293-NFκB-bsd-cTLR21 cell line can be used to identify oligonucleotides capable of eliciting a TLR21-mediated immune response.

[0108] Oligonucleotides and methods for their use in activating or otherwise stimulating TLR21 are also provided herein. In some embodiments the oligonucleotides comprise at least one pathogen associated molecular marker (PAMP), specifically an unmethylated dinucleotide CpG motif, which interacts with pathogen recognition receptors expressed in the host organism. In some embodiments, the oligonucleotides also have a guanine nucleotide enriched sequence. These sequences can facilitate the folding of a DNA strand into a quaternary structure or, in the case of oligonucleotides, promote the aggregation of one or more oligonucleotides comprising the sequence. It is demonstrated herein that the immunogenicity of oligonucleotides having CpG dinucleotide motifs can be enhanced if the oligonucleotide further comprises a guanine nucleotide enriched sequence. The guanine nucleotide enriched sequence need not be comprised solely of guanine nucleotides, but it must be enriched. A guanine-enriched sequence, as described above and as exemplified throughout these disclosures, is a segment of an oligonucleotide comprising more guanine nucleotides than any other residue (i.e., adenine, cytosine, thymine nucleotides). In some embodiments, additional manipulation of the oligonucleotide sequence and structure can further enhance the oligonucleotide’s ability to stimulate TLR21. Therefore, one embodiment of the

present disclosure comprises an oligonucleotide comprising at least one CpG motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

[0109] It has been previously shown that the addition of deoxyguanine (dG) nucleotides to the 3' end of a CpG containing oligonucleotide enhanced TLR9 activation *in vitro*. Because TLR9 is the mammalian functional equivalent of chicken TLR21, it was expected that 3' dG runs would also improve immunogenicity of oligonucleotides designed to activate TLR21. Surprisingly, this is not true for 3' guanine nucleotide enriched sequences in TLR21 activation. Oligonucleotides having 3' runs of two or more dGs failed to activate TLR21 (FIGs. 4A and 4B), whereas the addition of dG runs to the 5' end of the CpG containing oligonucleotide significantly improved immunogenicity of the oligonucleotide.

[0110] Not only does the position of the guanine nucleotide enriched sequence in the oligonucleotide affect enhancement of TLR21 activation, but the content of the sequence has an effect as well. For this reason, in some embodiments of the present disclosure, the guanine nucleotide 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.

[0111] In some embodiments of the present invention, an 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 some embodiments, the guanine nucleotide 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: 268), 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.

[0112] A single run of dG is not the only 5' modification that can enhance TLR21 stimulation. For example, adenine, cytosine, and thymine nucleotides enriched sequences can also be added to the 5' end of an oligonucleotide having at least one CpG motif and result in enhanced TLR21 stimulation, albeit less than that elicited by guanine-enriched sequences at the 5' end of the oligonucleotide (see FIGs. 8A and 8B). 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 nucleotide 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.

[0113] In some aspects, the 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, allow for interaction between oligonucleotides. Without being bound by theory, interaction of the oligonucleotides via G-quartet sequences allows for the concentration of CpG dinucleotide motifs and a corresponding enhanced probability of recognition by TLR21. G-quartet sequences also provide the opportunity for multiple TLR21 receptor interactions (enhancing avidity) and for receptor crosslinking. 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 (SEQ ID NO: 265).

[0114] A G-wire sequence is another guanine nucleotide enriched sequence that can be added to the 5' of an oligonucleotide having CpG motifs. 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 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.

[0115] Without being bound by any particular theory, it is possible that a G-wire sequence enables an oligonucleotide to interact, and aggregate, with other oligonucleotides having G-wire sequences. This accumulation of oligonucleotides and their CpG motifs may lead to enhanced stimulation of TLR21.

[0116] The guanine nucleotide enriched sequences within an oligonucleotide 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 nucleotide enriched sequence and the downstream at least one CpG motif. The linker need not be directly adjacent to either the guanine nucleotide enriched sequence or the CpG motif, but the linker must reside between the two sequence motifs regardless of intervening sequences between the guanine nucleotide 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. In some embodiments, the linker 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.

[0117] 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.

[0118] 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 motif—along with the CpG motif itself—constitute a CpG sequence element (e.g., XCGX, where X = any nucleotide). In some embodiments, the oligonucleotides of the present disclosure, comprise CpG sequence elements that are GCGA, GCGG, ACGC, CCGC, GCGT, TCGC, or any combination thereof.

[0119] 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.

[0120] In other embodiments of the presently disclosed oligonucleotides, each of the CpG motifs are separated from each 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.

[0121] 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 deoxyribosephosphate 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 deoxyribosephosphate bridge. The deoxyribosephosphate bridge may

comprise nitrogenous bases in some aspects while in others the deoxyribosephosphate bridge is abasic. In some aspects, the oligonucleotide comprises SEQ ID NO:221, which comprises an abasic deoxyribosephosphate bridge.

[0122] 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.

[0123] 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 1,3-propanediol; or 2006-PDE5dG4-X4, wherein X4 is a three carbon chain derived from 1,3-propanediol.

[0124] 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.

[0125] 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.

[0126] Although dG 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' terminus. In some aspects, the oligonucleotide comprises SEQ ID NO: 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, or 215.

[0127] For each oligonucleotide disclosed herein, one skilled in the art would know that a nucleotide can be substituted for a nucleotide analog. The oligonucleotides in some embodiments comprise a phosphodiester backbone, although other embodiments of the oligonucleotides disclosed herein comprise a phosphorothioate backbone.

[0128] 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, may lead to polydentate ligands via micelle or other aggregate formation, or any combination thereof. In some aspects, the lipid moiety is a cholesterol.

[0129] Because the oligonucleotides disclosed stimulate an enhanced immune response via TLR21, other embodiments of the present disclosure includes methods of preventing or treating disease by administering to a subject in need thereof a herein disclosed immunostimulatory oligonucleotide.

[0130] Also provided are immunostimulatory compositions comprising a herein disclosed immunostimulatory oligonucleotide. While these immunostimulatory compositions comprise an oligonucleotide as described herein, the compositions may also include other components that affect the immunogenicity, effectiveness, and efficiency of the composition. For example, in some aspects the immunostimulatory composition comprises a pharmaceutically acceptable 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 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.

[0131] In some embodiments, the oligonucleotide and the carrier are linked. As used to describe the relationship between the oligonucleotide and the carrier, “linked” refers to physical association of the oligonucleotide and the carrier. When the oligonucleotide and the carrier are bound to each other, interact with each other, or are combined, coupled, or otherwise joined, they can be deemed to be linked.

[0132] The immunostimulatory compositions described herein further comprise a hapten in some embodiments. In some aspects, the immunostimulatory oligonucleotide is linked to the hapten. The hapten may elicit an immunoresponse against a specific microorganism, such as, but not limited to, *E. coli* or *Salmonella*, while the immunostimulatory oligonucleotide elicits a non-specific immunoresponse mediated by TLR21 interaction with the oligonucleotide. These and other infectious microorganisms are of particular concern in large scale brooder houses in which the inhabitants are at increased risk of infection.

[0133] Some embodiments of the immunostimulatory compositions provide a vaccine for preventing or treating infectious disease comprising at least one of the immunostimulatory oligonucleotides described herein. For an oligonucleotide to elicit any immune response it must be effectively delivered to its target, whether the target is a cell culture, a chicken, or another vertebrate. Therefore, one aspect of the present disclosure provides a vector comprising an immunostimulatory oligonucleotide described herein.

[0134] The potency of the immunostimulatory oligonucleotide, and therefore immunostimulatory composition comprising only the oligonucleotide as an active ingredient, can be measured by its half-maximum effective concentration (EC₅₀). EC₅₀ is a measurement of the concentration of the immunostimulatory composition that induces 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 disclosure, the immunostimulatory composition can have an EC₅₀ in the pM range. In some aspects, the EC₅₀ is between about 0.1 and 100 pM. In some aspects, the EC₅₀ is between about 100 and 200 pM. In some aspects the EC₅₀ is between about 200 and 300 pM. In some aspects, the EC₅₀ is between about 300 and 400 pM. In some aspects the EC₅₀ is between about 400 and 500 pM. In some aspects the EC₅₀ is between about 500 and 600 pM. In some aspects the EC₅₀ is between about 600 and 700 pM. In some aspects the EC₅₀ is between about 700 and 800 pM. In some aspects the EC₅₀ is between about 800 and 900 pM. In some aspects the EC₅₀ is between about 900 pM and 1 nM. In still other aspects, the EC₅₀ is less than about 100 pM.

[0135] 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 1 μ M. In still other aspects, the concentration of the oligonucleotide is less than about 20 nM.

[0136] 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.

[0137] Also provided herein are methods of stimulating toll-like receptor 21 (TLR21) comprising administering to a subject in need thereof an oligonucleotide having at least one CpG motif and an guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide. Methods are also provided for eliciting an immune response in a

subject comprising administering to a subject in need thereof an immunostimulatory oligonucleotide having at least one CpG dinucleotide motif and at least one guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

[0138] The oligonucleotide can be administered in the form of an immunostimulatory composition as described above. The immunostimulatory composition may further comprise a hapten, a pharmaceutically acceptable carrier, or both, as described above. Administering the immunostimulatory composition, in some aspects, can be performed intravenously, intramuscularly, intramammary, intradermally, intraperitoneally, subcutaneously, by spray, by aerosol, in ovo, mucosally, transdermally, by immersion, orally, intraocularly, intratracheally, or intranasally. The subject in need of the administration is an animal. In some aspects, the subject is a member of an avian species. For example, the immunostimulatory composition disclosed herein may be administered in ovo to an embryonated chicken egg or intramuscularly to hatched chicks or even adult birds.

[0139] Also provided herein are methods for increasing TLR21-stimulatory activity of an oligonucleotide having at least one CpG motif, comprising fusing the 5' end of the oligonucleotide to a guanine nucleotide enriched sequence. In some aspects, the guanine nucleotide enriched sequence is a G-quartet sequence. In some aspects, the G-quartet sequence comprises a first plurality of guanine nucleotides. This first plurality of guanine nucleotides may comprise part of a TGGGGT sequence (SEQ ID NO: 265). In some aspects, the first plurality of guanine nucleotides comprises three to eight guanine nucleotides. In still other aspects, the G-quartet 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: 268), GGAGG, TGGAGGC, or TGGAGGCTGGAGGC (SEQ ID NO:264).

[0140] Other embodiments of the present disclosures provide for the guanine nucleotide enriched sequence to comprise a first and a second plurality of guanine nucleotides. In other aspects, the guanine nucleotide enriched sequence comprises a G-wire sequence. In some aspects, the G-wire sequence comprises SEQ ID NO:257 or 258. In still other aspects of the method, the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least one nucleotide.

[0141] The method as disclosed herein may further comprise, in some embodiments, inserting a linker between the first plurality of guanine nucleotides and the at least one CpG motif.

The linker has been described above and may comprise, but is not limited to, at least three nucleotides or a hexaethylene glycol.

[0142] The ability of an oligonucleotide to stimulate TLR21 may be further enhanced according to some aspect of the invention by increasing the number of CpG motifs in the oligonucleotide. 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 method disclosed provide for inserting at least one nucleotide or nucleotide analog between the CpG motifs. The at least one nucleotide may be two or three thymine nucleotides.

[0143] Other embodiments of the method provide for inserting 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 deoxyribosephosphate bridge, which can be abasic in some aspects.

[0144] 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.

[0145] Also envisioned in the method to enhance the TLR21 stimulatory properties of an oligonucleotide is incorporating at least one nucleotide analog or lipid moiety in the oligonucleotide. In some aspects, the lipid moiety is at or near the 5' terminus of the oligonucleotide. Still other embodiments of the method include modifying the nucleotides adjacent to the CpG motif.

EXAMPLES

[0146] 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.

Example 1: Generation of an NF κ B pathway reporter gene HEK293 cell line

[0147] pNifTy2-SEAP (Invivogen) and other commercially available plasmid vectors are routinely used to generate NFκB pathway reporter gene cell lines. The commercially available form of pNifTy2-SEAP comprises a zeocin resistance gene for bacterial and mammalian selection, which requires large amounts of this cytostatic (up to 400 μg/ml), and the selection is sometimes not reliable. Therefore, the generation of a reporter plasmid with better selection markers, preferably a blasticidin resistance, was initiated.

[0148] The open reading frame encoding the pNifty2-SEAP-encoded (secreted embryonic alkaline phosphatase) SEAP gene was synthesized in a human codon-optimized form. The 284 bp region in pNifty2-SEAP upstream the ATG start codon, which encompasses five NFκB recognition sites and an endothelial-leukocyte adhesion molecule (ELAM) promoter site, was also synthesized with the following modification: a KpnI site was constructed immediately upstream the ATG start codon (insertion of the sequence “GGTA”), and further upstream, a sequence was introduced consisting of 5’ to 3’ an EcoRV, an MluI, and an NdeI site. Furthermore, downstream from the stop codon, an NheI site and a second EcoRV site were introduced. Care was taken to avoid the presence of these sites in the SEAP open reading frame.

NFκB-SEAP (human codon-optimized) (SEQ ID NO:1) (Underlining shows restriction enzyme sites used for subcloning (MluI and NdeI). The start ATG and the stop TAA codons are emphasized in bold.)

GATATCACGCGTCAATTGGGATCTGCGATCGCTGAATTCTGGGGACTTTCCACTGGGGA
CTTTCCACTGGGGACTTTCCACTGGGGACTTTCCACTGGGGACTTTCCACTCCTGCAGCA
GTGGATATTCCCAGAAAACTTTTTGGATGCAGTTGGGGATTTCCTCTTTACTGGATGTG
GACAATATCCTCCTATTATTCACAGGAAGCAATCCCTCCTATAAAAGGGCCTCAGCAGA
AGTAGTGTTCACTGCTGTTCTTGGCTGACTTCACATCAAAGCTTCTATACTGACCTGAGAC
AGAGGGTACC**ATGGT**GCTGGGTCCATGCATGCTGCTGCTCCTTCTGCTGCTGGGACTTC
GATTGCAGCTGTCTCTGGGCATTATACCCGTTGAGGAAGAGAATCCAGACTTTTGGAAC
AGAGAAGCAGCCGAGGCGCTTGGAGCAGCTAAGAACTTCAACCAGCTCAGACTGCAG
CCAAGAACCTGATCATCTTCCTGGGCGATGGCATGGGTGTGTCAACGGTTACTGCCGCT
AGGATCCTGAAAGGCCAGAAGAAAGACAACTGGGTCCCGAAATTCTCTCGCCATGG
ACAGGTTCCCCTACGTTGCTCTGAGCAAGACCTATAATGTGGACAAGCACGTCCCAGAT
AGCGGAGCCACAGCTACCGCCTATCTGTGTGGTGTGAAGGGCAATTTTCAGACAATCG

GACTCTCCGCTGCCGCTCGGTTCAACCAGTGCAACACGACTAGGGGCAATGAGGTGATT
 TCCGTGATGAATCGCGCCAAGAAGGCGGGGAAAAGCGTAGGGGTGGTCACTACTACTC
 GGGTTCAGCACGCTTCTCCCGCCGGCACCTACGCTCACACCGTGAATCGAAACTGGTAC
 TCCGACGCTGACGTGCCGGCATCAGCACGGCAGGAAGGATGCCAAGACATCGCCACAC
 AGCTGATCAGTAACATGGACATAGACGTAATTCTGGGCGGTGGGCGGAAGTACATGTT
 TCGGATGGGGACTCCTGATCCCGAGTATCCCGACGACTACTCTCAGGGTGGTACACGAC
 TCGACGGCAAGAACCTGGTCCAGGAATGGCTTGCCAAGCGGCAAGGGGCGAGATACGT
 CTGGAATCGCACAGAACTGATGCAAGCCTCCTTGGATCCTTCCGTGACCCACTTGATGG
 GCTTGTTTGAGCCTGGGGATATGAAGTATGAGATCCACCGCGATTCTACCCTGGATCCT
 TCTCTGATGGAGATGACCGAAGCAGCCCTCAGGCTGCTGAGTCGGAATCCAAGGGGCT
 TCTTCTTGTTTCGTTGAGGGAGGCCGTATTGACCATGGGCACCATGAGTCAAGAGCGTAT
 AGAGCCCTCACCGAAACCATCATGTTTGACGATGCCATAGAGAGGGGCAGGACAGCTGA
 CGAGTGAGGAGGATACTCAGCCTGGTGACCGCAGATCACAGCCACGTCTTTAGCTTC
 GGCGGTTATCCGCTTCGTGGAAGCTCCATTTTCGGACTGGCACCAGGGAAAGCCAGAG
 ATCGCAAAGCTTACACAGTCCTCCTCTATGGAAACGGACCCGGGTATGTACTGAAAGAT
 GGCCTCGTCCGGACGTGACCGAGAGCGAATCAGGAAGTCCCGAATACAGGCAACAGT
 CCGCGGTTCCCCTTGATGAAGAGACTCACGCCGGGGAGGACGTGGCCGTGTTTGCGAG
 AGGGCCTCAGGCCCATCTCGTGTCATGGGGTACAGGAGCAGACATTTCATTGCCCATGTCA
 TGGCTTTTGCCGCCTGTCTGGAACCATAACGGCATGTGATCTGGCTCCTCCTGCTGGC
 ACAACCGATGCAGCACATCCAGGCAGATCTCGCAGCAAACGCTTGGACTGACTTAAGG
CTAGCGATATC

[0149] This synthetic SEAP gene construct (“NFκB-SEAP”) was excised from a cloning vector by MluI/NheI double digest and introduced by ligation into a pcDNA3.1(+) vector (FIG. 1), pre-cut with MluI/NheI and gel-purified to remove the CMV promoter region. Homemade versions of pcDNA3.1(+), where the neomycin resistance gene (NeoR/KanR) has been replaced by a blasticidin resistance gene (bsd → pcDNA3.1(+)-bsd) or a puromycin resistance gene (puro → pcDNA3.1(+)-puro) were processed in the same way and ligated with the NFκB-SEAP construct.

[0150] From this set of constructs, the bsd-containing plasmid pcDNA3.1(+)-bsd-NFκB-SEAP was chosen for HEK293 transfection. To this end, the PvuI-linearized form was introduced into the cells by standard transfection methods and cells with a genome-integrated construct were selected with 10 µg/ml blasticidin. Resistant cell pools were tested for tumor necrosis factor alpha

(TNF- α) induced SEAP production, and by single cell cloning, one clonal line (HEK293-NF κ B-SEAP-bsd or HEK293-NF κ B-bsd) with a particularly advantageous signal-to-noise ratio was chosen for further studies. The EC₅₀ for human TNF- α for this cell line is 3.2 ng/ml (FIG. 2).

Example 2: Generation of a chicken TLR21 transgenic cell line

[0151] Chicken toll-like receptor 21 (TLR21) is a non-methylated CpG DNA receptor that is functionally homologous, but not orthologous, to mammalian TLR9 (Brownlie et al. 2009, Kestra et al. 2010). The gene encoding chicken TLR21 was synthesized based on the deduced protein sequence of Genbank accession number NM_001030558 and by optimizing towards human codon usage. Upstream from the start codon ATG, a KpnI site including a Kozak sequence was introduced, while downstream from the stop codon a NotI site and an EcoRV site was added. The TLR21 gene was excised from the cloning vector by KpnI/NotI double digest, gel-purified and ligated into KpnI/NotI-cut mammalian expression vector pcDNA3.1(+). This pcDNA3.1(+)-cTLR21 was linearized with PvuI and transfected together with PvuI-linearized pcDNA3.1(+)-bsd-NF κ B-SEAP into HEK293 resulting in HEK293-NF κ B-bsd-cTLR21, or HEK293-bsd-cTLR21.

[0152] A cell pool was selected by simultaneous application of blasticidin and G418, tested for functional NF κ B pathway by TNF- α and for active cTLR21 by phosphorothioate oligonucleotide 2006-PTO (SEQ ID NO:3). Single cell cloning led to a clonal cell line with excellent signal-to-noise ratio in response to 2006-PTO. The clonal HEK293-NF κ B-bsd-cTLR21 cell line showed excellent TNF- α sensitivity (EC₅₀ = 1.4 ng/ml), akin to that observed for HEK293-NF κ B-bsd (FIG. 2).

***Gallus gallus* TLR21-Gen (based on NM_001030558) (SEQ ID NO:2)** (The start ATG and the stop TAG codons are emphasized by underlining.):

CCCGGTACCATGATGGAAACAGCTGAGAAAGCCTGGCCATCTACCAGGATGTGTCCTA
GTCAGTGTGCTCCCCCTCTGGCTGCTGCTGCTTGTACCGTGACGCTGATGCCAATGGTAC
ACCCTTATGGTTTCCGCAACTGCATCGAGGATGTCAAGGCTCCCTTGTACTTTAGGTGT
ATCCAGAGATTCTGCAGAGCCCAGCCCTCGCGGTGAGTGATCTTCCTCCCCATGCCAT
TGCTTGAAGTTGAGTTACAACAAGATGCGGTGTCTCCAGCCATCAGCCTTCGCCCACC
TGACGCAGTTGCATACGCTGGACCTGACTTACAATCTGCTCGAAACCCTGAGCCCTGGG
GCCTTCAATGGCTTGGGCGTCCTCGTGGTGCTCGACCTGTCTCACAATAAGCTGACTAC

TCTTGCAGAAGGGGTGTTTAACAGTCTGGGTAATCTGTCCTCCCTGCAAGTGCAGCATA
ACCCTCTGAGCACAGTCTCACCATCAGCACTTTTGCCACTGGTCAATCTCCGCAGGCTG
AGCCTGCGGGGAGGACGGCTGAATGGACTGGGCGCTGTTGCCGTGGCGGTTTCAGGGAC
TTGCACAGCTTGAGCTGCTGGATCTGTGTGAAAATAATTTGACAACACTGGGACCCGGT
CCGCCTCTGCCCCGCTAGCCTGCTCACCTGCAGCTGTGCAACAACACTCACTGAGGGAGCT
GGCCGGAGGAAGCCCTGAAATGCTGTGGCATGTGAAGATCCTGGATTTGTCATACAAC
AGCATCTCTCAGGCTGAAGTGTTTACTCAGCTCCACCTCCGCAATATCTCCCTTCTGCAC
TTGATTGGAAATCCCCTGGATGTGTTCCATTTGCTGGACATATCCGATATACAACCTAG
GTCCTGGACTTCTCAGGTCTGGTTCTTGGTGCCCAAGGGCTGGACAAGGTGTGTCTGC
GTCTGCAAGGGCCCCAGGCTCTTCGCCGTCTGCAACTTCAGAGAAACGGGGCTCAAAGTC
CTGCACTGCAACGCCCTGCAGCTTTGCCCCGTGCTGCGAGAGCTGGATCTGTCTTGAA
CCGCCTGCAGCACGTGCGCTGTGCAGGCCGACTCCTCGGGAAGAAACAGCGGGAGAAA
CTGGAAGTTCTGACCGTGGAACACAATCTTCTGAAGAACTCCCCAGTTGCTTGGGTGC
CCAAGTGCTCCCTAGACTGTATAACGTCAGCTTCCGGTTCAATCGAATCCTGACTGTGG
GTCCACAGGCCTTCGCCTATGCACCCGCGCTCCAGGTCCTTTGGCTGAACATTAACCTCC
CTTGTCTGGTTGGATCGTCAGGCTCTTTGGCGCCTCCATAATCTGACCGAGCTGAGACTT
GATAACAATCTGTTGACAGATCTGTACCACAACCTCTTTCATTGACCTTCACAGACTGCG
GACCCTGAATCTCCGGAACAACCGCGTGAGCGTTCTGTTTTCCGGGGTTTTCCAGGGCT
TGGCCGAGCTGCAGACCCTGGACCTGGGCGGCAACAATCTGCGACACCTCACAGCTCA
GAGTCTGCAGGGCCTCCCAAAGCTGAGGAGGCTGTACCTCGACCGGAATAGACTTCTG
GAGGTGTCCTCAACTGTATTTGCTCCCGTTCAAGCCACCCTCGGGGTGCTGGACCTGAG
AGCCAACAATCTGCAGTATATCTCCAGTGCGCTTAGGAAACCGCCGCCATTTAGAAACT
TGAGCAGCCTGTATGACCTGAAACTGCAGGCCCAGCAGCCGTATGGGCTGAAGATGCT
GCCTCACTACTTCTTTCAGGGCCTGGTTAGACTGCAACAGCTCTCCCTTAGCCAAAACA
TGCTGAGGTCTATCCCACCGGACGTGTTTGAAGATCTCGGACAGCTCCGTAGCCTGGCT
CTGGCTGACAGTAGCAATGGGCTGCATGATTTGCCCGACGGCATTTCGGAACCTCGG
GAACCTGAGGTTTCTCGATCTTGAGAATGCGGGGTGCACTCTCTCACCTGGAGGTCT
TTGGAAACCTCTCCCGCCTGCAAGTCCTGCATCTGGCAAGGAACGAACCTCAAAACCTTC
AATGACTCTGTGGCAAGCCGGCTGAGCAGCCTTCGCTATCTGGACCTCCGGAAGTGTCC
TCTGTCTTGCACTTGCGATAATATGTGGCTGCAGGGGTGGTTGAATAATTCTCGGGTAC
AGGTAGTGTACCCCTACAACCTACACATGCGGATCTCAACACAACGCATACATACACAG
CTTTGACACACATGTCTGCTTTCTGGATCTGGGCTTGTACTTGTTTCGAGGCACCGCTCC

TGCTGTACTGCTCCTCCTCGTCGTACCCGTAGTATATCATCGCGCATACTGGCGGTTGAA
 GTACCACTGGTATCTTCTGAGATGTTGGGTGAATCAGCGCTGGAGAAGGGAGGAAAAG
 TGCTATCTGTATGACTCATTGTCTCTTACAACAGTGCGGATGAGTCCTGGGTTTTGCAA
 AAGCTCGTCCCAGAGCTCGAGCATGGGGCCTTCAGATTGTGTCTCCATCACAGGGACTT
 CCAGCCAGGAAGGAGTATTATCGACAATATCGTGGATGCGGTTTATAACAGTCGTAAA
 ACGGTGTGCGTTGTGTCAAGATCCTACCTTAGATCCGAGTGGTGCAGCCTCGAGGTGCA
 GCTGGCATCCTATCGACTTCTGGATGAGCGCCGAGACATTTTGGTGCTGGTGCTGCTGG
 AGGATGTGGGTGACGCCGAGCTGAGCGCATATCATCGCATGAGGAGAGTGCTGCTGAG
 GCGCACATACCTCCGGTGGCCTCTGGATCCAGCCGCTCAACCCCTGTTTTGGGCTAGAT
 TGAAACGAGCCCTTCGATGGGGCGAGGGCGGAGAAGAGGAGGAAGAAGAAGGTCTGG
 GAGGCGGCACTGGCCGGCCTCGTGAAGGCGACAAGCAGATGTAGCGGCCGCGATATC

[0153] The phosphorothioate (PTO) oligodeoxynucleotide (ODN) 2006-PTO (ODN 2006) is known to activate TLR21. Kestra, A.M., de Zoete, M.R., Bouwman, L.I., van Putten, J.P., 2010. Chicken TLR21 is an innate CpG DNA receptor distinct from mammalian TLR9. *J. Immunol.* 185, 460-467. In the clonal TLR21 cell line of this study (HEK293-NFκB-bsd-cTLR21), 2006-PTO was also active, with an EC₅₀ of activation of ~ 8.5 nM. By contrast the HEK293-NFκB-bsd did not show any SEAP secretion (FIG. 3A). This demonstrates the specific interaction of this ODN is specifically on TLR21. 2006-PDE, the phosphodiester-bonded version of 2006-PTO, was much weaker in its stimulatory activity on TLR21. An estimate for its EC₅₀ is > 250 nM, with much lower maximum stimulation compared to 2006-PTO (FIG. 3B).

Example 3: ODN comprising 5' G-quartet forming sequences enhance TLR21 activity

Impact of 3'-deoxyguanine (dG) additions on TLR21 recognition of 2006-PDE (ODN2006, phosphodiester form)

[0154] The phosphodiester-bonded version of 2006-PTO, 2006-PDE, was used as a basis to investigate the effect of 3'-dG modification on the TLR21-stimulatory activity in the HEK293-NFκB-bsd-cTLR21 cell line described in Example 2. To this end, titration experiments were performed starting at 20 nM with 15 dilution steps (1:2) reaching approximately 1 pM as a final dilution. 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 NaHCO₃/Na₂CO₃, 2 mM MgCl₂, 5mM *para*-nitrophenylphosphate (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).

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

2006-PTO	SEQ ID NO:3	tcgtcggttttgcggttttgcggtt
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

[0155] While as expected, 2006-PTO stimulated TLR21 in the nanomolar range, 2006-PDE showed no significant TLR21-stimulatory activity. Addition of one dG at the 3' end led to some marked TLR21-stimulatory activity in the nM range, which was still present with a second (dG2) and a third (dG3) dG addition, albeit much weaker. Addition of a 4th, 5th, 6th, 7th and 8th dG (dG4-dG8) resulted in TLR21 inactive ODNs (FIG. 4A). In the concentration range up to 0.33 nM (330 pM), none of the ODNs showed TLR21 activity (FIG. 4B).

Impact of 5'dG additions on TLR21 recognition of 2006-PDE (ODN2006, phosphodiester form)

[0156] The phosphodiester-bonded version of 2006-PTO, 2006-PDE, was used as a basis to investigate the effect of 5'-dG modification on the TLR21-stimulatory activity. To this end, titration experiments were performed starting at 20 nM with 15 dilution steps (1:2) reaching approximately 1 pM as a final dilution.

Table 2: ODN sequences (lower case: PTO bonds, upper case PDE bonds)

2006-PTO	SEQ ID NO:3	tcgtcgttttgcgttttgcgtt
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

[0157] 2006-PTO stimulated TLR21 in the nanomolar range and 2006-PDE showed no significant TLR21-stimulatory activity. Addition of one dG and two Gs at the 5'-end of 2006-PDE led to some minor TLR21-stimulatory activity in the double digit nM range. A third dG (dG3) led to a dramatic increase of TLR21 activity, with a calculated EC₅₀ of 513 picoMolar (pM) (Table 3). Addition of a 4th dG further 14-fold increased activity (calculated EC₅₀ of 36 pM, Table 3), while a 5th, 6th, 7th and 8th dG (dG4-dG8) resulted in a further EC₅₀ increase and a TLR21 stimulatory plateau with EC₅₀'s between 17.1 and 22.2 pM. Taken together, it appears that after the addition of 3dGs, but not yet two dGs, at the 5' end, some fundamental change in ODN structure happens, that leads to a massive increase of TLR21 activity, from almost inactivity to strong picomolar activity, that is further increased by additional 5' dGs. The equivalent additions of dGs at the 3' end do not lead to high activity, the corresponding ODN derivatives are largely inactive (compare FIGs. 4A-B, 5A-B, and 6, as well as Table 3).

Table 3: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE	Largely inactive	Largely inactive
2006-PTO	22463	1223
2006-PDE3dG1	35072	1121
2006-PDE3dG2	Largely inactive	Largely inactive

2006-PDE3dG3	Largely inactive	Largely inactive
2006-PDE3dG4	inactive	inactive
2006-PDE3dG5	inactive	inactive
2006-PDE3dG6	inactive	inactive
2006-PDE3dG7	inactive	inactive
2006-PDE3dG8	inactive	Inactive
2006-PDE5dG1	weak	weak
2006-PDE5dG2	Largely inactive	Largely inactive
2006-PDE5dG3	513	589
2006-PDE5dG4	36.0	559
2006-PDE5dG5	22.2	553
2006-PDE5dG6	17.1	549
2006-PDE5dG7	22.2	555
2006-PDE5dG8	21.9	559

[0158] To investigate the electrophoretic migration behavior of the ODNs tested on TLR21, 16% TBE polyacrylamide gel electrophoresis was performed, followed by methylene blue staining. In the case of 2006-PDE-5dG0-8, there is a clear correlation between the appearance of higher order structures (FIGs. 7A, 7B) and high TLR21 stimulatory activity. It appears likely, that the higher order structures are formed by the generation of G-quartet structures known to be formed often by consecutive Gs, and potentially involving the same strand ('intramolecular G-quartets') or different strands ('intermolecular G-quartets') of DNA. Williamson JR, *G-Quartet Structures in Telomeric DNA*, Ann. Rev. Biophys. Biomol. Struct., 23: 703-730 (1994); Simonsson T, *G-Quadruplex DNA Structures – Variations on a Theme*, Biol. Chem. 382: 621-628 (2001). However, the same aggregation is observed in the 2006-PDE-3dG0-8 oligonucleotides, which are poorly active or inactive on TLR21. This suggests that aggregation alone is not sufficient for strong TLR21 stimulatory activity. Positioning of the consecutive guanines to the 5' end appears to impact TLR21 stimulatory activity.

Further examination of the dependence on 5' guanine runs of the potent TLR21 stimulation using the example 2006-PDE-5dG6.

[0159] 2006-PDE-5dG6 was picked as an example, because it appeared to be forming the plateau of TLR21 stimulatory activity in the 5'dG_n scan of 2006-PDE (see FIGs. 5A, 5B, 6, and Table 3). The 5'-dG₆ run was replaced by dA₆, dT₆, or dC₆ (Table 4).

Table 4:

2006-PDE	SEQ ID NO: 4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dA6	SEQ ID NO:22	AAAAAATCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dC6	SEQ ID NO:23	CCCCCCTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dT6	SEQ ID NO:24	TTTTTTTCGTCGTTTTGTCGTTTTGTCGTT

[0160] When 2006-PDE is modified with dN₆ at the 5' end, every base homomer yields some increase of TLR21 activity in the order of improvement: dA₆ < dC₆ < dT₆ <<<<<<<<< dG₆ (FIG. 8A) The improvement of 5'-dG₆ is clearly orders of magnitude higher than that of the other bases (visible in particular at low concentrations, FIG. 8B), suggesting a special quality conferred by this modification with G-quartet-forming potential.

Examination of the dependence on the presence of CpG elements of the potent TLR21 stimulation by 5'-dG-modified 2006-PDE using the example 2006-PDE-5dG6.

[0161] 2006-PDE-5dG6 was picked as an example, because it appeared to be forming the plateau of TLR21 stimulatory activity in the 5'dG_n scan of 2006-PDE (see FIGs. 5A, 5B, 6, and Table 3). The impact of the CpG motifs on the TLR21 stimulatory activity were investigated by 1) synthesizing this ODN with 5-methyl-cytidine replacing every cytidine in the four CpG motifs, by 2) inverting every CpG motif to GpC, and by 3) replacing every guanine in the CpG motifs with adenine, by replacing every cytosine with thymine, and by simultaneous replacement of cytosine and guanine with thymine and adenine, respectively. The resulting oligonucleotides were tested for their ability to stimulate TLR21 using HEK293-NFκB-bsd-cTLR21 cells as described in Example 3.

Table 5:

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT

2006-PDE5dG6-Me	SEQ ID NO:25	GGGGGGTXGTGXGTTTTGTGXGTTTTGTGXGTT
2006-PDE5dG6-GC	SEQ ID NO:26	GGGGGGTGCTGCTTTTGTGCTTTTGTGCTT
2006-PDE5dG6-CA	SEQ ID NO:27	GGGGGGTCATCATTTTGTGCTTTTGTGCTT
2006-PDE5dG6-TG	SEQ ID NO:266	GGGGGGTTGTTGTTTGTGTTTGTGTTGTT
2006-PDE5dG6-TA	SEQ ID NO:267	GGGGGGTTATTATTTTGTATTATTGTATT
X = 5 methyl cytidine		

[0162] Every modification investigated here that interferes with the integrity of the CpG motifs in 2006-PDE-5dG6 leads to a massive loss of activity (FIG. 9A), that becomes particularly visible at low ODN concentrations (FIG. 9B).

Examination of the impact of 3'- and 5'-dG6 modifications of 2006-PTO on TLR21 stimulatory activity and comparison to equivalent changes in 2006-PDE.

[0163] To investigate whether the TLR21-stimulatory activity improvement by dG run addition also applies to oligodeoxynucleotides with phosphorothioate backbone (PTO-ODNs), the PTO congeners of 2006-PDE, 2006-PDE-3dG5 and 2006-PDE-5dG6 were synthesized (Table 6), and their ability to stimulate TLR21 using HEK293-NFκB-bsd-cTLR21 cells as described in Example 3 was compared with each other and their PDE-versions (Table 7, FIGs. 10A and 10B).

Table 6: ODN sequences PTO *versus* PDE (PTO lower case)

2006-PTO	SEQ ID NO:3	tcgtcgttttgcgttttgcgtt
2006-PTO3dG5	SEQ ID NO:28	tcgtcgttttgcgttttgcgttggggg
2006-PTO5dG6	SEQ ID NO:29	ggggggtcgtcgttttgcgttttgcgtt
2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE3dG5	SEQ ID NO:9	TCGTCGTTTTGTCGTTTTGTCGTTGGGGG
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT

Table 7: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE	Largely inactive	-

2006-PDE3dG5	Largely inactive	-
2006-PDE5dG6	17.1	556
2006-PTO	22463	
2006-PTO3dG5	106775	2162
2006-PTO5dG6	42.7	655

[0164] In the absence of 5'dG residues, PTO modification confers much higher activity to ODNs compared to the PDE versions (Table 7, FIGs. 10A and 10B). This is different for 5'dG6-modified 2006-PDE compared to its PTO version. Here, PDE does confer even slightly higher activity (EC50), which is unexpected (Table 7, FIGs. 10A and 10B).

Examination of the impact of dA replacements in the 5dG6 run of 2006-PDE-5dG6 on TLR21 stimulatory activity.

[0165] Based on the hypothesis that the consecutive dG sequences 5' of 2006-PDE form G-quartets conferring TLR21-stimulatory activity, it was predicted that dA replacements in a dG6 run expected to disrupt G-quartet formation should have, depending on the position, a negative impact on TLR21 stimulatory activity. To this end, single and double dA replacement 2006-PDE-5dG6 ODNs were synthesized using methods familiar to those in the art and tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Tables 8 and 9, FIGs. 11A-D).

Table 8: ODN sequences, A replacements

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
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	GGGAATCGTCGTTTTGTCGTTTTGTCGTT

Table 9: Effective concentration 50% (EC₅₀) and the maximal signal (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE	Largely inactive	Largely inactive
2006-PDE5dG6	17.1	556
2006-PDE5dG6-A1	29.0	593
2006-PDE5dG6-A2	97.1	570
2006-PDE5dG6-A3	206	584
2006-PDE5dG6-A4	318	568
2006-PDE5dG6-A5	47.0	559
2006-PDE5dG6-A6	22.6	549
2006-PDE5dG6-A12	69.1	551
2006-PDE5dG6-A23	11705	460
2006-PDE5dG6-A34	7849	760
2006-PDE5dG6-A45	113	539
2006-PDE5dG6-A56	35.0	500

[0166] In general, all dA replacements within the dG6 run led to little changes in V_{max} (i.e., the maximal reporter gene readout obtained in comparative experiments), while the EC₅₀ varied considerable up to more than two orders of magnitude (Table 9 and FIGs. 11A and 11B). Single replacements in the 1st and 6th positions were very mild on the EC₅₀, while the 2nd and 5th position led to a more pronounced increase. The strongest changes were observed for the 3rd and 4th positions, which led to a more than 10-fold increase in EC₅₀. In the case of double dA replacements (Table 9, FIGs. 11C and 11D), the consecutive 1st and 2nd as well as the 5th and 6th led to relatively mild EC₅₀ increases, while 4th and 5th led to a more strong increase. Double dA replacement of the 2nd and 3rd, as well as of the 3rd and 4th positions led to increases of EC₅₀ of 685-fold and 459-fold, respectively. Given the fact that 3 consecutive dGs have been identified before in this study as the

minimum number for potent TLR21 activity and EC₅₀ increases were noted in the order dG3, dG4 to dG5, after which an EC₅₀ plateau was seen from dG5-dG8 (compare FIGs 5A, 5B, 6, and Table 3), these data further support the notion that the undisturbed formation of G-quartets 5' of 2006-PDE is a prerequisite for strong TLR21 stimulation.

Examination of the impact of dC replacements in the 5dG6 run of 2006-PDE-5dG6 on TLR21 stimulatory activity.

[0167] Based on the hypothesis that the consecutive dG sequences 5' of 2006-PDE form G-quartets conferring TLR21-stimulatory activity, it was predicted that dC replacements in a dG6 run expected to disrupt G-quartet formation should have, depending on the position, a negative impact on TLR21 stimulatory activity. To this end, single and double dC replacement 2006-PDE-5dG6 ODNs were synthesized and tested for their ability to stimulate TLR21 as explained in Example 3. (Table 10, Table 11, FIGs. 12A-D).

Table 10: ODN sequences, C replacements

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
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-C5	SEQ ID NO:45	GGGGCGTCGTCGTTTTGTCGTTTTGTCGTT
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

Table 11: Half-maximum effective concentration (EC₅₀) and the maximal signal (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min
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		(mOD405/min)
2006-PDE	Largely inactive	Largely inactive
2006-PDE5dG6	17.1	556
2006-PDE5dG6-C1	35.7	498
2006-PDE5dG6-C2	43.5	494
2006-PDE5dG6-C3	295	546
2006-PDE5dG6-C4	301	531
2006-PDE5dG6-C5	44.0	480
2006-PDE5dG6-C6	35.9	480
2006-PDE5dG6-C12	83.5	486
2006-PDE5dG6-C23	2738	473
2006-PDE5dG6-C34	5176	578
2006-PDE5dG6-C45	813	552
2006-PDE5dG6-C56	62.6	544

[0168] In general, all dC replacements within the dG6 run led to little changes in Vmax (i.e., the maximal reporter gene readout obtained in comparative experiments), while the EC₅₀ varied considerable up to more than two orders of magnitude (Table 11 and FIGs. 12A and 12B). Single replacements in the 1st and 6th positions of the oligonucleotide were very mild on the EC₅₀, as were the 2nd and 5th position of the oligonucleotide. The strongest changes were observed for the 3rd and 4th positions of the oligonucleotide, with led to a more than 10-fold increase in EC₅₀. In the case of double dC replacements (Table 11 and FIGs. 12C and 12D), the consecutive 1st and 2nd as well as the 5th and 6th positions of the oligonucleotide led to relatively mild EC₅₀ increases, while 4th and 5th positions led to a more strong increase. Double dC replacement of the 2nd and 3rd positions, as well as of the 3rd and 4th positions of the oligonucleotide led to massive increases of EC₅₀ of 160-fold and 303-fold, respectively. Given that 3 consecutive dGs have been identified in this study as the minimum number for potent TLR21 activity and EC₅₀ increases were noted in the order dG3, dG4 to dG5, after which an EC₅₀ plateau was seen from dG5-dG8 (compare FIGs. 5A-B, 6, and Table 3), these data further support the notion that the undisturbed formation of G-quartets 5' of 2006-PDE is a prerequisite for strong TLR21 stimulation.

Examination of the impact of dT replacements in the 5dG6 run of 2006-PDE-5dG6 on TLR21 stimulatory activity.

[0169] Based on the hypothesis that the consecutive dG sequences at the 5' terminus of 2006-PDE form G-quartets conferring TLR21-stimulatory activity, it was predicted that dT replacements in a dG6 run expected to disrupt G-quartet formation should have, depending on the position, a negative impact on TLR21 stimulatory activity. To this end, single and double dT replacement 2006-PDE-5dG6 ODNs were synthesized and tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 12, Table 13, FIG. 13A-D).

Table 12: ODN sequences, T replacements

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6	SEQ ID NO:19	GGGGGGTCGTCGTTTTGTCGTTTTGTCGTT
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	GGGTGTGTCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG6-T56	SEQ ID NO:62	GGGGTTTCGTCGTTTTGTCGTTTTGTCGTT

Table 13: Half-maximum effective concentration (EC₅₀) and the maximal signal (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE	Largely inactive	Largely inactive
2006-PDE5dG6	17.1	556
2006-PDE5dG6-T1	17.6	495

2006-PDE5dG6-T2	36.9	500
2006-PDE5dG6-T3	128	521
2006-PDE5dG6-T4	138	539
2006-PDE5dG6-T5	16.4	511
2006-PDE5dG6-T6	26.7	562
2006-PDE5dG6-T12	37.1	536
2006-PDE5dG6-T23	30459	629
2006-PDE5dG6-T34	10639	636
2006-PDE5dG6-T45	572	565
2006-PDE5dG6-T56	31.2	514

[0170] In general, all dT replacements within the dG6 run led to little changes in V_{max} , while the EC_{50} varied considerably, up to more than three orders of magnitude (Table 13 and FIGs. 13A and 13B). Single replacements in the 1st and 6th positions of the oligonucleotide were very mild on the EC_{50} , as were the 2nd and 5th position of the oligonucleotide. The strongest changes were observed for the 3rd and 4th positions of the oligonucleotide, with led to a more than 6-fold increase in EC_{50} . In the case of double dT replacements (Table 13 and FIGs. 13C and 13D), the consecutive 1st and 2nd as well as the 5th and 6th positions of the oligonucleotide led to relatively mild EC_{50} increases, while 4th and 5th positions led to a more strong increase. Double dT replacement of the 2nd and 3rd positions of the oligonucleotide, as well as of the 3rd and 4th positions led to massive increases of EC_{50} of 1781-fold and 622-fold, respectively. Given that three consecutive dGs have been identified in this study as the minimum number for potent TLR21 activity and that EC_{50} increases were noted in the order dG3, dG4 to dG5, after which an EC_{50} plateau was seen from dG5-dG8 (compare FIGs. 5A, 5B, 6, and Table 3), these data further support the notion that the undisturbed formation of G-quartets 5' of 2006-PDE is a prerequisite for strong TLR21 stimulation.

[0171] FIG. 14 illustrates that dG replacements at positions 1 and 6 of the oligonucleotide are rather benign. By contrast, any replacement at positions 3 and 4 of the oligonucleotide does have marked negative effects of TLR21 stimulatory potential. It also appears that adjacent dGdG double replacements at positions 1 and 2, as well as 5 and 6, of the oligonucleotide are benign. By contrast, FIG. 15 illustrates that at positions 2 and 3 as well as 3 and 4 of the oligonucleotide, replacement of adjacent dGdG by any homodinucleotid (dCdC, dAdA, and particularly dTdT) leads to a dramatic

loss of TLR21 stimulatory activity. Adjacent dGdG double replacements at positions 4 and 5 of the oligonucleotide are more moderate, but also lead to loss of activity.

[0172] These data indicate that a consecutive run of four dGs is essential for high TLR21 stimulatory activity and disruption of the four dG run by any other nucleotide results in a dramatic loss of activity.

Examination of the impact of dA replacements in the 5dG4 run of 2006-PDE-5dG4 on the TLR21 stimulatory activity.

[0173] To more stringently test the hypothesis that 5'-dG4 is necessary and sufficient to confer high level stimulatory activity to 2006-PDE, dG moieties in 2006-PDE-5dG4 were systematically replaced by dA and the various derivatives were tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 14, Table 15, FIGs. 16A and 16B).

Table 14: ODN sequences, A replacements

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4	SEQ ID NO:17	GGGGTCGTCGTTTTGTCGTTTTGTCGTT
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

Table 15: Half-maximum effective concentration (EC₅₀) and the maximal signal (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE5dG4	35.3	519
2006-PDE5dG4-A1	Weakly active	-
2006-PDE5dG4-A2	12066	1017
2006-PDE5dG4-A3	16640	1149
2006-PDE5dG4-A4	548	684

[0174] All dA replacements within the dG4 run led to losses of TLR21 stimulatory activity. Somewhat surprising, a dramatic change in TLR21 stimulatory activity was noted in position 1, to the extent that an EC₅₀ could not be calculated (Table 15 and FIGs. 16A and 16B). Single dA replacements in the 2nd and 3rd positions of 2006-PDE5dG4 led also to massive increases of EC₅₀, with factors of 342 and 471, respectively. The mildest loss of activity was observed in dA replacement of position 4 in the dG4 run, with an EC₅₀ increase of factor 16 (Table 15 and FIGs. 16A and 16B). These data further support the notion that the undisturbed formation of G-quartets 5' of 2006-PDE is a prerequisite for strong TLR21 stimulation.

Examination of the impact of dC replacements in the 5dG4 run of 2006-PDE-5dG4 on TLR21 stimulatory activity.

[0175] To test more stringently the hypothesis that 5'-dG4 is necessary and sufficient to confer high level stimulatory activity to 2006-PDE, dG moieties in 2006-PDE-5dG4 were systematically replaced by dC and the various derivatives were tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 16, Table 17, FIGs. 17A and 17B).

Table 16: ODN sequences, C replacements

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4	SEQ ID NO:17	GGGGTCGTCGTTTTGTCGTTTTGTCGTT
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

Table 17: Half-maximum effective concentration (EC₅₀) and the maximal signal (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE5dG4-N2	35.3	519
2006-PDE5dG4-C1	3153	764
2006-PDE5dG4-C2	29357	1361

2006-PDE5dG4-C3	19228	1229
2006-PDE5dG4-C4	515	669

[0176] All dC replacements within the dG4 run led to losses of TLR21 stimulatory activity. A marked loss in TLR21 stimulatory activity was noted in position 1, with an EC₅₀ increase of factor 89 (Table 17 and FIGs. 17A and 17B). Single dC replacements in the 2nd and 3rd positions led also to massive increases of EC₅₀, with factors of 831 and 545, respectively. The mildest loss of activity was found in dC replacement of position 4 in the dG4 run, with an EC₅₀ increase of factor 15 (Table 17 and FIGs. 17A and 17B). These data further support the notion that the undisturbed formation of G-quartets at the 5' terminus of 2006-PDE is a prerequisite for strong TLR21 stimulation.

Examination of the impact of dT replacements in the 5dG4 run of 2006-PDE-5dG4 on TLR21 stimulatory activity.

[0177] To test more stringently the hypothesis that the 5'-dG4 motif is necessary and sufficient to confer high level stimulatory activity to 2006-PDE, dG moieties in 2006-PDE-5dG4 were systematically replaced by dT and the various derivatives were tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 18, Table 19, FIGs. 18A and 18B).

Table 18: ODN sequences, A replacements

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE5dG4	SEQ ID NO:17	GGGGTCGTCGTTTTGTCGTTTTGTCGTT
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

Table 19: Half-maximum effective concentration (EC₅₀) and the maximal signal (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
-----	---------------------------------	--

2006-PDE5dG4	35.3	519
2006-PDE5dG4-T1	Weakly active	-
2006-PDE5dG4-T2	3232	733
2006-PDE5dG4-T3	9337	961
2006-PDE5dG4-T4	191	605

[0178] All dT replacements within the dG4 run led to losses of TLR21 stimulatory activity. A somewhat surprising and dramatic change in TLR21 stimulatory activity was observed for position 1, to the extent that an EC₅₀ could not be calculated (Table 19 and FIGs. 18A and 18B). Single dA replacements in the 2nd and 3rd positions led also to massive increases of EC₅₀, with factors of 92 and 265, respectively. The mildest loss of activity was found in dT replacement of position 4 in the dG4 run, with an EC₅₀ increase of factor 5 (Table 19 and FIGs. 18A and 18B). These data further support the notion that the undisturbed formation of G-quartets 5' of 2006-PDE is a prerequisite for strong TLR21 stimulation.

[0179] As FIG. 19 illustrates, any dG replacements in the dG4 run are detrimental to TLR21 stimulatory activity, which is in-line with the view that four consecutive dGs are required for high activity. Interestingly, any replacement in position 1 eliminated TLR21 activity despite preserving three consecutive dGs, while replacement of position 4 dG, which also preserves three consecutive dGs, was comparatively benign. Replacement of dG2 or dG3 was uniformly detrimental to TLR21 activity.

Impact of 5'-dG4 and 5'-dG6 addition to CpG-containing PDE-ODNs on conferring TLR21 stimulatory activity.

[0180] Eleven CpG-containing oligodeoxynucleotide sequences implicated in the literature as stimulatory for mammalian TLR9 (but mostly described as PTO versions) were chosen for synthesis as PDE derivatives (Table 20). TLR21 interaction was unknown for all these PDE ODNs. For five of these PDE ODNs, their 3'-dG5-, 5'-dG4- and 5'-dG6-versions were also synthesized. For one ODN, its 5'-dG4- and 5'-dG6-versions were synthesized; for another, its 3'-dG5- and 5'-dG6-versions were synthesized; while for 4 others, only the corresponding 5'-dG6-versions were synthesized (Table 20). These ODNs were subjected to TLR21 stimulation testing as described in Example 3 (Table 21, FIGs. 20A-I).

Table 20: ODNs used for dG_n attachment

ODN	SEQ ID NO	Sequence
1668	SEQ ID NO:75	TCCATGACGTTTCCTGATGCT
1668-3dG5	SEQ ID NO:76	TCCATGACGTTTCCTGATGCTGGGGG
1668-5dG4	SEQ ID NO:77	GGGGTCCATGACGTTTCCTGATGCT
1668-5dG6	SEQ ID NO:78	GGGGGGTCCATGACGTTTCCTGATGCT
1826	SEQ ID NO:79	TCCATGACGTTTCCTGACGTT
1826-3dG5	SEQ ID NO:80	TCCATGACGTTTCCTGACGTTGGGGG
1826-5dG4	SEQ ID NO:81	GGGGTCCATGACGTTTCCTGACGTT
1826-5dG6	SEQ ID NO:82	GGGGGGTCCATGACGTTTCCTGACGTT
BW006	SEQ ID NO:83	TCGACGTTTCGTCGTTTCGTCGTTTC
BW006-3dG5	SEQ ID NO:84	TCGACGTTTCGTCGTTTCGTCGTTTCGGGGG
BW006-5dG4	SEQ ID NO:85	GGGGTCGACGTTTCGTCGTTTCGTCGTTTC
BW006-5dG6	SEQ ID NO:86	GGGGGGTCGACGTTTCGTCGTTTCGTCGTTTC
D-SLO1	SEQ ID NO:87	TCGCGACGTTTCGCCCCGACGTTTCGGTA
D-SLO1-3dG5	SEQ ID NO:88	TCGCGACGTTTCGCCCCGACGTTTCGGTAGGGGG
D-SLO1-5dG4	SEQ ID NO:89	GGGGTCGCGACGTTTCGCCCCGACGTTTCGGTA
D-SLO1-5dG6	SEQ ID NO:90	GGGGGGTCGCGACGTTTCGCCCCGACGTTTCGGTA
2395	SEQ ID NO:91	TCGTCGTTTTTCGGCGCGCGCCG
2395-5dG4	SEQ ID NO:92	GGGGTCGTCGTTTTTCGGCGCGCGCCG
2395-5dG6	SEQ ID NO:93	GGGGGGTCGTCGTTTTTCGGCGCGCGCCG
M362	SEQ ID NO:94	TCGTCGTCGTTTCGAACGACGTTGAT
M362-3dG5	SEQ ID NO:95	TCGTCGTCGTTTCGAACGACGTTGATGGGGG
M362-5dG4	SEQ ID NO:96	GGGGTCGTCGTCGTTTCGAACGACGTTGAT
M362-5dG6	SEQ ID NO:97	GGGGGGTCGTCGTCGTTTCGAACGACGTTGAT
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	SEQ ID NO:102	GGGGGGGATCTCGCTCGCTCGCTAT
CPG-685	SEQ ID NO:103	TCGTCGACGTCGTTTCGTTCTC

CPG-685-5dG6	SEQ ID NO:104	GGGGGGTCGTCGACGTCGTTTCGTTCTC
CPG-2000	SEQ ID NO:105	TCCATGACGTTTCCTGCAGTTCCTGACGTT
CPG-2000-5dG6	SEQ ID NO:106	GGGGGGTCCATGACGTTTCCTGCAGTTCCTGACGTT
CPG-2002	SEQ ID NO:107	TCCACGACGTTTTCGACGTT
CPG-2002-5dG6	SEQ ID NO:108	GGGGGGTCCACGACGTTTTCGACGTT

Table 21: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
1668	inactive	-
1668-3dG5	Inactive	-
1668-5dG4	15140	71
1668-5dG6	6328	45
1826	minor activity	-
1826-3dG5	Inactive	-
1826-5dG4	866	309
1826-5dG6	478	373
BW006	minor activity	-
BW006-3dG5	Inactive	-
BW006-5dG4	20.3	378
BW006-5dG6	76	311
D-SLO1	some activity	-
D-SLO1-3dG5	Inactive	-
D-SLO1-5dG4	174	372
D-SLO1-5dG6	129	372
M362	Inactive	-
M362-3dG5	Inactive	-
M362-5dG4	6832	609
M362-5dG6	38480	1214
2395	Inactive	-
2395-5dG4	121	410

2395-5dG6	1092	475
2007	minor activity	-
2007-3dG5	Inactive	-
2007-5dG6	20.3	637
202	Inactive	-
202-5dG6	92.2	446
685	Inactive	-
685-5dG6	97.4	451
2000	Inactive	-
2000-5dG6	530	727
2002	Inactive	-
2002-5dG6	61.6	833

[0181] In TLR21 activation tests of the unmodified PDE ODNs (Table 20), only 1826, BW006, D-SLO1 and 2007 showed some minor activity (Table 21, FIGs. 20B, 20C, 20D, 20G). All other PDE ODNs exhibited no TLR21 stimulatory activity at the concentration tested (Table 21, FIGs. 20A, 20E, 20F, 20H, 20I, 20J, and 20K). The six ODN derivatives having addition of 3'dG5 did not exhibit TLR21 activity (Table 21). This is in line with the earlier observations of 2006-PDE. For the four ODNs with minor TLR21 stimulation signal (1826, BW006, D-SLO1, and 2007), 3'dG5 addition killed their activity (FIGs. 20B, 20C, 20D, 20G).

[0182] In contrast, addition of 5'dG4 (six ODNs) or 5'dG6 (eleven ODNs) led to increased TLR21 stimulatory activity in each case including nanomolar EC₅₀'s in five cases picomolar (pM) EC₅₀ in thirteen other cases (Table 21). Six even had double digit pM EC₅₀ (as low as 20 pM), which is highly remarkable, given that the starting point was near zero. Taken together, the data suggests that potent TLR21 activity can be achieved by attaching dG runs to the 5'-end (but NOT the 3'-end) of previously poorly active or inactive CpG-containing PDE-ODNs.

[0183] The data presented here also suggests that the activity gain is connected to the intermolecular formation by the 5'dG_n-modified ODNs of a G-quartet DNA superstructure.

Impact of 5'dG additions on mouse TLR9 recognition of 2006-PDE

[0184] The phosphodiester bond version of 2006-PTO, 2006-PDE, was used as a basis to investigate the effect of 5'-dG modification on the stimulatory activity on murine TLR9 and human

TLR9. Titration experiments were performed starting at 2000 nM or 5000 nM ODN concentration with 15 dilution steps (1:2) reaching approximately 100 pM or 500 pM as final dilutions. Derivative ODNs of 2006-PTE are described in Table 21-2.

Table 21-2: Derivative ODNs of 2006-PDE (PTO bonds lower case)

2006-PTO	SEQ ID NO: 3	tcgtcgttttgcgttttgcgtt
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

[0185] 2006-PTO stimulated mouse and human TLR9 in the nanomolar range. 2006-PDE showed only minor mouse or human TLR9-stimulatory activity (FIGs. 68A and 68B). Addition of one to eight dGs at the 5'-end of 2006-PDE led to no or only minor increases of activity of mouse TLR9 (FIG. 68A). In human HEKBlue cells, addition of one to six dG at the 5' end of 2006-PDE led to no increase or even a decrease in stimulatory activity. Addition of dG7 and dG8 at the 5' end of the oligonucleotide having CpG motifs led to some minor increase in activity of human TLR9 (FIG. 68B). Collectively, this picture is in stark contrast to the observation that chicken TLR21 stimulatory activity of 2006-PDE is strongly boosted by the addition of three to eight dGs at the 5' end of the oligonucleotide.

Impact of 3' dG additions on mouse and human TLR9 recognition of 2006-PDE

[0186] Addition of one to three dGs at the 3' end of 2006-PDE led minor progressive increases of activity in mouse TLR9 (FIG. 69A). Addition of a fourth dG at the 3' end of the oligonucleotide led to a strong increase in mouse TLR9 stimulatory activity, which was slightly improved or preserved upon addition of a 5th, 6th, 7th or 8th 3' dG to the 3' end of the oligonucleotide (FIG. 69A).

[0187] For stimulation of human TLR9, addition of one to three dG at the 3' end of 2006-PDE led to marked progressive increase in stimulatory activity relative to the parental 2006-PDE. Addition of a fourth dG at the 3' end of the oligonucleotide led to a strong increase in human TLR9

stimulatory activity. This stimulatory effect was slightly improved or preserved upon addition of a 5th, 6th, 7th or 8th 3' dG (FIG. 69B).

[0188] Collectively, this picture is in stark contrast to the observation that chicken TLR21 stimulatory activity of 2006-PDE is not boosted or even decreased by the addition of 3-8 dGs at the 3' end. Taken together, the structure-activity relationships for 5'-dG and 3'-dG additions on 2006-PDE with respect to TLR stimulatory activity are fundamentally different for mouse and human (and presumably mammalian) TLR9 compared to chicken (and presumably bird) TLR21. This may reflect the fact that TLR21 is only a functional, but not a true genetic, ortholog of TLR9 in birds.

Example 4: Sequences known or suspected to form G-quartet structures confer TLR21 stimulatory activity when linked to the 5' end of largely inactive 2006-PDE.

[0189] **Test phase I.** A number of telomere and promoter sequence elements with proposed G-quartet-forming potential were added to the 5' end of 2006-PDE. Additionally, 5' T4-modified 2006-PDE (2006-PDE-T4) was used to determine the ability of HEK293-NFκB-bsd-cTLR21 cells to stimulate TLR21 as described in Example 3 (Table 22).

Table 22: ODN sequences (Underlining indicates sequences considered to be involved in G-quartet formation)

<i>ODNs forming the basis and standards</i>	SEQ ID NO	Sequence
2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-5dG4	SEQ ID NO:17	<u>GGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006-T4-PDE	SEQ ID NO:109	TTTTTCGTCGTTTTGTCGTTTTGTCGTT
<i>ODN fusions derived from telomeres:</i>		
2006-HuTel-1	SEQ ID NO:110	<u>TTAGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006-HuTel-2	SEQ ID NO:111	<u>TTAGGGTTAGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Oxy1	SEQ ID NO:112	<u>TTTTGGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT

2006-PDE-Oxy2	SEQ ID NO:113	<u>GGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>
2006-PDE-Oxy3	SEQ ID NO:114	<u>GGGGTTTTGGGGTCGTCGTTTTGTCGTTTTGTCGTT</u>
2006-T4-HuTel-1	SEQ ID NO:115	<u>TTAGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>
2006-T4-HuTel-2	SEQ ID NO:116	<u>TTAGGGTTAGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>
2006-T4-ScerTel	SEQ ID NO:117	<u>TGTGGGTGTGTGTGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>
<i>Derived from a promoter:</i>		
2006-T4-cMyc	SEQ ID NO:118	<u>GGAGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>
2006-T4-cMyc2	SEQ ID NO:119	<u>TGGAGGCTTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>
2006-T4-cMyc3	SEQ ID NO:120	<u>TGGAGGCTGGAGGCTTTTTTCGTCGTTTTGTCGTTTTGTCGTT</u>

Table 23: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
2006-PDE	Inactive	-
2006-HuTel-1	12611	269
2006-HuTel-2	1374	271
2006-T4-PDE	weakly active	-
2006-T4-HuTel-1	4563	284
2006-T4-HuTel-2	769	270
2006-T4-ScerTel	152	341
2006-T4-cMyc	28.8	318
2006-T4-cMyc2	1190	366
2006-T4-cMyc3	436	359

[0190] Fusion of human telomere sequences to 2006-PDE and 2006-PDE-T4 resulted in ODNs capable of activating TLR21 with nanomolar (nM) EC₅₀ or even picomolar (pM) activity (Table 23, FIG. 21A). The yeast telomere sequence conferred high TLR21 activity, with an EC₅₀ as low as 152 pM. The c-myc-promoter-derived sequences tested were also capable of activating

2006-PDE-T4 towards TLR21 stimulation, one derivative yielding a double digit pM activity (Table 23, FIG. 21B and 21C).

[0191] 2006-PDE fusions with sequence elements of *Oxytricha spp.* telomeres (a preferred early model species for telomere research, and for G-quartet structure research) were synthesized (Table 22) and tested for their TLR21 stimulatory potential (Table 24, FIGs. 22A and 22B). In this study, the fused sequences comprised the following: TTAGGG, TTAGGGTTAGGG (SEQ ID NO:261), TTTTGGGG, GGGGTTTT, GGGGTTTTGGGG (SEQ ID NO:262), TTAGGG, TTAGGGTTAGGGTTTT (SEQ ID NO:263), TGTGGGTGTGTGTGGG (SEQ ID NO: 268), GGAGG, TGGAGGC, or TGGAGGCTGGAGGC (SEQ ID NO:264).

Table 24: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE	Inactive	-
2006-PDE-5dG4	54.7	668
2006-PDE-Oxy1	40.2	650
2006-PDE-Oxy2	19.3	638
2006-PDE-Oxy3	48.5	609

[0192] The *Oxytricha spp.* telomere sequence elements conferred highly potent TLR21 activity to inactive 2006-PDE. The resulting derivatives were amongst the most potent derivatives identified to this point.

[0193] **Test phase II.** 20 different promoter elements shown or predicted to involve G-quartet formation were selected, and 5' fusion constructs comprising 2006-PDE and the promoter elements were synthesized (Table 25) for testing in HEK293-NFκB-bsd-cTLR21 cells to determine their ability to stimulate TLR21.

Table 25: ODN sequences (Underlining indicates sequences considered to be involved in G-quartet formation)

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-	SEQ ID	<u>GGGGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT

5dG6	NO:19	
EA2-2006	SEQ ID NO:121	<u>GCTGCGAGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT</u>
EA2D-2006	SEQ ID NO:122	<u>GCTGCGGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT</u>
EA2a-2006	SEQ ID NO:123	<u>CGAGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT</u>
EA2aD- 2006	SEQ ID NO:124	<u>CGGGCGGGTGGGTGGGATCGTCGTTTTGTCGTTTTGTCGTT</u>
HCV-2006	SEQ ID NO:125	<u>GGGCGTGGTGGGTGGGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>
HIV-93del- 2006	SEQ ID NO:126	<u>GGGGTGGGAGGAGGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>
Hema-2006	SEQ ID NO:127	<u>GGGGTCGGGCGGGCCGGGTGTCGTCGTTTTGTCGTTTTGTCGTT</u>
Insu-2006	SEQ ID NO:128	<u>GGTGGTGGGGGGGGTTGGTAGGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>
IonK-2006	SEQ ID NO:129	<u>GGGTAGGGTTAGGGTAGGGTCGTCGTTTTGTCGTTTTGTCGTT</u>
Scle-2006	SEQ ID NO:130	<u>TGGGGGGGTGGGTGGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>
STAT-2006	SEQ ID NO:131	<u>GGGCGGGCGGGCGGGCTCGTCGTTTTGTCGTTTTGTCGTT</u>
TBA-2006	SEQ ID NO:132	<u>GGTTGGTGTGGTTGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>
TNF-2006	SEQ ID NO:133	<u>GGTGGATGGCGCAGTCGGTCGTCGTTTTGTCGTTTTGTCGTT</u>
apVEGF-D- 2006	SEQ ID NO:134	<u>TGGGGGTGGACGGGCCGGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>
apVEGF- 2006	SEQ ID NO:135	<u>TGTGGGGGTGGACGGGCCGGGTTCGTCGTTTTGTCGTTTTGTCGTT</u>

HTR-2006	SEQ ID NO:136	<u>GGGTTAGGGTTAGGGTTAGGGTCGTCGTTTTGTCGTTTTGTCGTT</u>
bcl-2-2006	SEQ ID NO:137	<u>GGGCGCGGGAGGAAGGGGGCGGGTCGTCGTTTTGTCGTTTTGTCGTT</u>
c-myc-2006	SEQ ID NO:138	<u>AGGGTGGGGAGGGTGGGGATCGTCGTTTTGTCGTTTTGTCGTT</u>
c-kit87- 2006	SEQ ID NO:139	<u>AGGGAGGGCGCTGGGAGGAGGGTCGTCGTTTTGTCGTTTTGTCGTT</u>
vegf-2006	SEQ ID NO:140	<u>GGGGCGGGCCGGGGGCGGGGTCGTCGTTTTGTCGTTTTGTCGTT</u>

Table 26: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE	inactive	0
2006-PDE-5dG6	29.4	452
EA2-2006	22.2	306
EA2D-2006	53.7	316
EA2a-2006	21.7	315
EA2aD-2006	55.1	313
HCV-2006	18.5	329
HIV-93del-2006	21.9	364
Hema-2006	22.4	402
Insu-2006	20.7	371
IonK-2006	377.0	386
Scle-2006	15.6	353
STAT-2006	30.6	355
TBA-2006	1172.0	434
TNF-2006	226.0	394

apVEGF-D-2006	19.4	373
apVEGF-2006	23.4	460
HTR-2006	421.0	438
bcl-2-2006	40.0	387
c-myc-2006	23.6	406
c-kit87-2006	23.3	403
vegf-2006	48.2	413

[0194] The TLR21 assay revealed that all elements tested conferred activity to the inactive 2006-PDE. Eleven of the twenty elements tested showed potencies exceeding that of TLR21 agonist, 2006-5dG6, with EC₅₀ values below 30 pM, and as low as 15.6 pM (Table 26).

Example 5: Identification, application, and optimization of new sequence elements and biophysical principles conferring TLR21 stimulatory activity to CpG-ODNs.

[0195] G-quartets (FIG. 23A) can be formed intramolecularly or intermolecularly and in parallel or antiparallel orientation (FIGs. 23B, 23C). Based on the hypothesis that G-quartet-mediated aggregation of ODNs leads to increased TLR21 stimulatory activity by generating a ligand variant with multiple binding sites for TLR21 (leading to interaction avidity gains and to receptor clustering), it was further hypothesized that optimizing orientation and binding site multiplicity should further enhance activity.

[0196] **Test phase I.** A number of telomere and promoter sequence elements with proposed G-quartet-forming potential were fused to the 5' end of 2006-PDE and 2006-PDE-T4 for testing in HEK293-NFκB-bsd-cTLR21 cells to determine their ability to stimulate TLR21 (Table 27).

[0197] Particularly interesting in this respect is the formation of a polymeric G-quartet structure from ODN monomers, called a G-wire (FIGs. 23C and 23D), as it does have the potential to generate a polymeric TLR21 ligand. Marsh TC, Vesenska J, Henderson E, *A New DNA Nanostructure, the G-wire, Imaged by Scanning Probe Microscopy*, Nucl. Acid Res., 23: 696-700 (1995). Specifically, 2006-PDE having the sequence GGGGTTGGGG (SEQ ID NO:257) fused to its 5' end appears to have the propensity to form such structures (Table 27). Because the

arrangement of CpG-ODN “actives” too close to such a polymer formed by 5' GGGGTTGGGG (SEQ ID NO: 257) is likely to lead to steric problems such as receptor interaction, derivatives with a dT4 spacer were also synthesized (Table 27).

[0198] It was previously reported, that the G-rich hexanucleotide TGGGGT (SEQ ID NO: 265) preferentially forms parallel-oriented tetrameric G-quartet structures (Phillips K, Dauter Z, Murchie AI, Lilley DM, Luisi BJ, *The Crystal Structure of a Parallel-Stranded Guanine Tetraplex at 0.95 Å Resolution*, J. Mol Biol. 273: 171-182 (1997)), (FIG. 23B). Such a tetrameric arrangement of CpG-containing ODNs linked by a 5'-parallel G-quartet is expected to provide an advantageous ligand arrangement for TLR21. Such a derivative of 2006-PDE was synthesized (Table 27), and tested, together with the above derivatives in comparison to 2006-PDE-5dG4 and 2006-PDE-5dG6 (Table 28, FIGs. 24A and 24B).

Table 27: ODN sequences (Underlining indicates sequences considered to be involved in G-quartet formation)

2006-PDE	SEQ ID NO:4	TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-5dG4	SEQ ID NO:17	<u>GGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-5dG6	SEQ ID NO:19	<u>GGGGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Gwire1	SEQ ID NO:141	<u>GGGGTTGGGG</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006-PDE-Gwire2	SEQ ID NO:142	<u>GGGGTTGGGGTTTT</u> TCGTCGTTTTGTCGTTTTGTCGTT
2006PDE5dG4-T1-6	SEQ ID NO:143	<u>TGGGGT</u> TCGTCGTTTTGTCGTTTTGTCGTT

Table 28: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
2006-PDE	inactive	-
2006-PDE-5dG4	58.0	692
2006-PDE-5dG6	29.4	603
2006-PDE-Gwire1	108	662
2006-PDE-Gwire2	19.2	593
2006PDE5dG4-T1-6	12.1	583

[0199] Addition of GGGGTTGGGG (SEQ ID NO: 257) to the 5' end of TLR21-inactive 2006-PDE (2006-PDE-Gwire1) led to an ODN with pM EC₅₀, but its activity fell short of the EC₅₀'s of 2006-PDE-5dG4 and 2006-PDE-5dG6, which were used as benchmarks in this study (Table 28, FIGs. 24A and 24B). However, introduction of 4 dT nucleotides between the G-wire sequence and 2006-PDE (2006-PDE-Gwire2) improved the EC₅₀ by a factor of 5 and yielded an activity superior

to the benchmarks (Table 28, FIGs. 24A and 24B). Addition of TGGGGT (SEQ ID NO: 265) to the 5' end of 2006-PDE resulted in an ODN with the lowest EC₅₀ for TLR21 to this point (Table 28, FIGs. 24A and 24B). The formation of higher order structures by 5'-G-wire modification was demonstrated by polyacrylamide gel electrophoresis (FIG. 25).

[0200] Taken together, two superior, presumably G-quartet sequence elements leading to potent TLR21 stimulatory activity of 2006-PDE were identified in this study. Without being bound by theory, it is likely that the potent TLR21 activating capacity of GGGGTTGGGG (SEQ ID NO: 257) is related to its known potential to form so-called G-wire structures (FIGs. 23C and 23D), providing a polydentate ligand with an advantageous orientation. It is also likely that the potent TLR21 activating capacity of TGGGGT (SEQ ID NO: 265) is related to its known potential to form parallel tetrameric intermolecular G-quartets, providing a tetradentate ligand with advantageous orientation (FIG. 23B).

[0201] Test phase II. The potential for TLR21 stimulation-enhancing activity of the benchmark sequence GGGGGG was tested and compared with the G-wire sequence GGGGTTGGGGTTTT (SEQ ID NO:258), that proved to be superior in the preceding study. To this end, 16 oligonucleotides were designed that were of the general sequence TTTTTTXXCGXTT (SEQ ID NO:259), where X represented any base (Table 29). The dTs were used to generate an oligonucleotide of acceptable length (14 bases), to encase the tetranucleotide CpG “warhead” in an ODN context, because it generates no problems in synthesis, and because of its low propensity to form unwanted secondary structures. Such short ODNs with only one CpG element and with PDE bonds are expected to be of low TLR21 stimulatory activity. Hence, the starting concentration for testing on TLR21 was raised 50-fold from 20 nM to 1000 nM. These 16 ODNs were also synthesized having 5'-GGGGGG termini and 5'-GGGGTTGGGGTTTT (Gwire2; SEQ ID NO: 258) termini (Table 29) and tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21.

Table 29: ODN sequences (Underlining indicates sequences considered to be involved in G-quartet formation and/or G-wire formation)

	Basal ODNs (All CpG-containing tetranucleotides)	
1-ACGA	TTTTTTTACGATTT	SEQ ID NO:144
2-GCGA	TTTTTTTGCGATTT	SEQ ID NO:145
3-CCGA	TTTTTTTCCGATTT	SEQ ID NO:146
4-TCGA	TTTTTTTTCGATTT	SEQ ID NO:147

5-ACGG	TTTTTTTACGGTTT	SEQ ID NO:148
6-GCGG	TTTTTTTGCGGTTT	SEQ ID NO:149
7-CCGG	TTTTTTTCCGGTTT	SEQ ID NO:150
8-TCGG	TTTTTTTTCGGTTT	SEQ ID NO:151
9-ACGC	TTTTTTTACGCTTT	SEQ ID NO:152
10-GCGC	TTTTTTTGCGCTTT	SEQ ID NO:153
11-CCGC	TTTTTTTCCGCTTT	SEQ ID NO:154
12-TCGC	TTTTTTTTCGCTTT	SEQ ID NO:155
13-ACGT	TTTTTTTACGTTTT	SEQ ID NO:156
14-GCGT	TTTTTTTGCGTTTT	SEQ ID NO:157
15-CCGT	TTTTTTTCCGTTTT	SEQ ID NO:158
16-TCGT	TTTTTTTTCGTTTT	SEQ ID NO:159
	Basal ODNs (All CpG-containing tetranucleotides)	
17-ACGA-5dG6	<u>GGGGGG</u> TTTTTTTACGATTT	SEQ ID NO:160
18-GCGA-5dG6	<u>GGGGGG</u> TTTTTTTGCGATTT	SEQ ID NO:161
19-CCGA-5dG6	<u>GGGGGG</u> TTTTTTTCCGATTT	SEQ ID NO:162
20-TCGA-5dG6	<u>GGGGGG</u> TTTTTTTTCGATTT	SEQ ID NO:163
21-ACGG-5dG6	<u>GGGGGG</u> TTTTTTTACGGTTT	SEQ ID NO:164
22-GCGG-5dG6	<u>GGGGGG</u> TTTTTTTGCGGTTT	SEQ ID NO:165
23-CCGG-5dG6	<u>GGGGGG</u> TTTTTTTCCGGTTT	SEQ ID NO:166
24-TCGG-5dG6	<u>GGGGGG</u> TTTTTTTTCGGTTT	SEQ ID NO:167
25-ACGC-5dG6	<u>GGGGGG</u> TTTTTTTACGCTTT	SEQ ID NO:168
26-GCGC-5dG6	<u>GGGGGG</u> TTTTTTTGCGCTTT	SEQ ID NO:169
27-CCGC-5dG6	<u>GGGGGG</u> TTTTTTTCCGCTTT	SEQ ID NO:170
28-TCGC-5dG6	<u>GGGGGG</u> TTTTTTTTCGCTTT	SEQ ID NO:171
29-ACGT-5dG6	<u>GGGGGG</u> TTTTTTTACGTTTT	SEQ ID NO:172
30-GCGT-5dG6	<u>GGGGGG</u> TTTTTTTGCGTTTT	SEQ ID NO:173
31-CCGT-5dG6	<u>GGGGGG</u> TTTTTTTCCGTTTT	SEQ ID NO:174
32-TCGT-5dG6	<u>GGGGGG</u> TTTTTTTTCGTTTT	SEQ ID NO:175
	5'-Gwire2-modified basal ODNs	
33-ACGA-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTACGATTT	SEQ ID NO:176
34-GCGA-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTGCGATTT	SEQ ID NO:177
35-CCGA-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTCCGATTT	SEQ ID NO:178
36-TCGA-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTTCGATTT	SEQ ID NO:179
37-ACGG-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTACGGTTT	SEQ ID NO:180
38-GCGG-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTGCGGTTT	SEQ ID NO:181
39-CCGG-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTCCGGTTT	SEQ ID NO:182
40-TCGG-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTTCGGTTT	SEQ ID NO:183
41-ACGC-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTACGCTTT	SEQ ID NO:184
42-GCGC-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTGCGCTTT	SEQ ID NO:185
43-CCGC-Gwire2	<u>GGGGTTGGGG</u> TTTTTTTCCGCTTT	SEQ ID NO:186

44-TCGC-Gwire2	<u>GGGGTTGGGGTTTTTTTTTTTCGCTTT</u>	SEQ ID NO:187
45-ACGT-Gwire2	<u>GGGGTTGGGGTTTTTTTTTTTACGTTTT</u>	SEQ ID NO:188
46-GCGT-Gwire2	<u>GGGGTTGGGGTTTTTTTTTTTGCGTTTT</u>	SEQ ID NO:189
47-CCGT-Gwire2	<u>GGGGTTGGGGTTTTTTTTTTTCCGTTTT</u>	SEQ ID NO:190
48-TCGT-Gwire2	<u>GGGGTTGGGGTTTTTTTTTTTTCGTTTT</u>	SEQ ID NO:191
Gwire2	<u>GGGGTTGGGGTTTT</u>	SEQ ID NO:258

[0202] The sixteen 14-mer ODNs comprising all potential permutations of tetranucleotides with a central CpG were largely inactive on TLR21 up to 1000 nM concentration, with the exception of the ACGC-containing and the CCGC-containing species (ODNs 9 and 11, respectively), which showed a detectable signal at the highest concentration.

[0203] Addition of dG6 (GGGGGG) to the 5' end of basal ODNs (SEQ ID NOs:144-159) led to some TLR21 stimulatory activity in most cases, with the exception of CCGA (ODN 19), CCGG (ODN 23), GCGC (ODN 26), and CCGT (ODN 31) (FIGs. 26B, 27B, 28B, and 29B). This confirms the potential of 5'-GGGGGG to confer TLR21 activity to CpG ODNs, although with the exception of GCGG (ODN 22), ACGA (ODN 25) and TCGC (ODN 28), the signal strength for each was weak (FIGs. 27B and 28B).

[0204] Addition of Gwire2 (GGGGTTGGGGTTTT (SEQ ID NO:258)) to the 5' end of basal ODNs (SEQ ID NOs:144-159) led to TLR21 stimulatory activity in all the cases, where 5dG6 succeeded, and in addition for CCGA (ODN 35), CCGG (ODN 39) and GCGC (ODN 42), while CCGT (ODN 47) remained refractory (FIGs. 26C, 27C, 28C, and 29C and 29D). However, the signal strength obtained with Gwire2 attachment was far higher than that seen with 5dG6. This was particularly evident for GCGA (ODN 18 *versus* ODN 34 (FIGs. 26B and 26C, respectively), GCGG (ODN 22 *versus* ODN 38 (FIGs. 27B and 27C, respectively)), ACGC (ODN 25 *versus* ODN 41 (FIGs. 28B and 28C, respectively)), CCGC (ODN 27 *versus* ODN 43 (FIGs. 28B and 28C, respectively)), TCGC (ODN 28 *versus* ODN 44 (FIGs. 28B and 28C, respectively)), and GCGT (ODN 30 *versus* ODN 46 (FIGs. 29B and 29C, respectively)). The latter ODN, 46, GCGT-Gwire2 was the most remarkable species: it exhibited outstanding TLR21 stimulatory activity already at picomolar concentrations, and the EC₅₀ could be determined as close to 2 nM (FIG. 29D).

[0205] The CpG-containing sequence elements GCGA, GCGG, ACGC, CCGC GCGT, and perhaps also TCGC, have not previously been described in the context of TLR21 activation.

[0206] **XCGA series.** None of the 14-mers from the XCGA series shows any TLR21 activity up to 1000 nM. Addition of 5'-dG6 leads to some activity of GCGA>ACGA>TCGA,

while CCGA remains inactive. Most remarkably, addition of 5'-Gwire2 leads to TLR21 activity for all four derivatives. A dramatic increase in TLR21 activity is noted for GCGA, while the others have increased activity in the relative order TCGA>ACGA>CCGA (FIGs. 26A-C).

[0207] XCGG series. Two of the 14-mers from the XCGG series show minor, if any, TLR21 activity at 1000 nM (GCGG, TCGG), while the other two oligonucleotides are inactive. Addition of 5'-dG6 leads to some activity of GCGG>ACGG>TCGG, while CCGG remains inactive. Most remarkably, addition of 5'-Gwire2 leads to TLR21 activity for all four derivatives. A dramatic increase in TLR21 activity is noted for GCGG, while the others have increased activity in the relative order TCGG>ACGG>CCGG (FIGs. 27A-C).

[0208] XCGC series. Two of the 14-mers from the XCGC series show minor, if any, TLR21 activity at 1000 and 500 nM (ACGC, CCGC), while the other two are inactive. Addition of 5'-dG6 leads to strong activity of TCGC>ACGC>CCGC, while GCGC remains inactive. Most remarkably, addition of 5'-Gwire2 leads once again to TLR21 activity for all four derivatives. A massive increase in TLR21 activity is noted for TCGC>ACGC>CCGC, in that order of activity, while GCGC remains weak (FIGs. 28A-C).

[0209] XCGT series. None of the 14-mers from the XCGT series shows any TLR21 activity up to 1000 nM (FIG. 29A). Addition of 5'-dG6 leads to some activity of all four, in the activity order TCGT>GCGT>ACGT>CCGT (FIG. 29B). Most remarkably, addition of 5'-Gwire2 leads to a dramatic increase in TLR21 activity for GCGT and activity of TCGT is also larger than noted for TCGT-5dG6 (FIG. 29C). Activity of Gwire2-modified ACGT and CCGT is no larger than for the 5dG6 derivatives (FIGs. 29C and 29D). The Gwire2 14mer ODN alone (GGGGTTGGGGTTTT (SEQ ID NO:258)), that is attached to the basal ODNs, is inactive on TLR21 (see Table 49, FIG. 29).

Example 6: The backbone of the most potent sequence is GCGT-Gwire2: Structure-activity relationships (SAR)

[0210] The investigations of SAR of GCGT-Gwire2 included modifying the central CpG element by inversion (GC), and pyrimidine-pyrimidine (TG) as well as purine-purine (CA) exchange (Table 30). Testing of these derivatives in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 revealed a complete loss of activity after these manipulations (Table 31, FIG. 30), confirming that the potent TLR21 stimulatory activity of GCGT-Gwire2 is crucially dependent on the presence of this single CpG element.

Table 30: ODN sequences

GCGT-Gwire2	SEQ ID NO:189	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-GC	SEQ ID NO:192	GGGGTTGGGGTTTTTTTTTTTGGCTTTT
GCGT-Gwire2-TG	SEQ ID NO:193	GGGGTTGGGGTTTTTTTTTTTGTGTTTT
GCGT-Gwire2-CA	SEQ ID NO:194	GGGGTTGGGGTTTTTTTTTTTGCATTTT
GCGT-Gwire2	SEQ ID NO:189	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-T1	SEQ ID NO:195	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-T2	SEQ ID NO:196	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-T3	SEQ ID NO:197	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-T4	SEQ ID NO:198	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-T5	SEQ ID NO:199	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-T6	SEQ ID NO:200	GGGGTTGGGGTTTTTTTCGTTTT
GCGT-Gwire2	SEQ ID NO:189	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire2-eT1	SEQ ID NO:201	GGGGTTGGGGTTTTTTTTTTTTCGTTT
GCGT-Gwire2-eT2	SEQ ID NO:202	GGGGTTGGGGTTTTTTTTTTTTCGTT
GCGT-Gwire2-eT3	SEQ ID NO:203	GGGGTTGGGGTTTTTTTTTTTTCGT
GCGT-Gwire2	SEQ ID NO:189	GGGGTTGGGGTTTTTTTTTTTTCGTTTT
GCGT-Gwire3	SEQ ID NO:224	GGGGTTGGGGTTGGGGTTTTTTTTTTTTCGTTTT

[0211] The number of dTs between the Gwire2 element and the GCGT element was also decreased (Table 30), and the corresponding ODNs were tested:

Table 31: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
GCGT-Gwire2	2886	272
GCGT-Gwire2-GC	inactive	-
GCGT-Gwire2-TG	inactive	-
GCGT-Gwire2-CA	inactive	-
GCGT-Gwire2	2886	272
GCGT-Gwire2-T1	1710	283
GCGT-Gwire2-T2	4194	286
GCGT-Gwire2-T3	7874	226
GCGT-Gwire2-T4	2477	278
GCGT-Gwire2-T5	8881	195
GCGT-Gwire2-T6	6527	136
GCGT-Gwire2	2886	272
GCGT-Gwire2-eT1	2459	168
GCGT-Gwire2-eT2	9422	27

GCGT-Gwire2-eT3	inactive	-
GCGT-Gwire2	2886	272
GCGT-Gwire3	373	344

[0212] The results from deletions T1-T4 on GCGT-Gwire2 led to little changes in V_{max} , and, except for T3, largely similar EC_{50} values. However, T5 and T6 deletions led to increased EC_{50} , and, particularly, a decrease in V_{max} , suggesting a loss of intrinsic activity (Table 31, FIG. 31).

[0213] For a third SAR study, the number of Ts flanking the GCGT element at the 3'-end of the ODN was decreased (Table 30), and the corresponding ODNs were tested. The effects were immediately obvious. While loss of one T (eT1) led to a decreased V_{max} under preservation of the EC_{50} , loss of two Ts (eT2) increased EC_{50} and dramatically reduced V_{max} , loss of three dTs eliminated the activity altogether (Table 31, FIG. 32).

[0214] In a fourth experiment, the effect of incorporating an additional GGGGT motif (GCGT-Gwire3, Table 30) on the intrinsic TLR21-stimulatory activity of GCGT-Gwire2 was investigated. The activity of GCGT-Gwire3 was superior to that of the parental GCGT-Gwire2 (Table 31, FIGs. 33A and 33B). The EC_{50} was 8-fold lower and the V_{max} also increased (Table 31). Preliminary SAR results of immunostimulatory GCGT-Gwire2 oligonucleotides is illustrated in FIG. 34.

Example 7: The influence of CpG element copy number on the TLR21 stimulatory activity of selected XCGX-Gwire2 species

Table 32: ODN sequences (Underlining indicates XCGX elements.)

GCGT-Gwire2	SEQ ID NO:189	GGGGTTGGGGTTTTTTTTTTT <u>GCGTTTT</u>
GCGT-Gwire2- do	SEQ ID NO:204	GGGGTTGGGGTTTTTTTTTTT <u>GCGTTTTGCGTTTT</u>
GCGT-Gwire2- tri	SEQ ID NO:205	GGGGTTGGGGTTTTTTTTTTT <u>GCGTTTTGCGTTTTGCGTTTT</u>
GCGA-Gwire2	SEQ ID NO:177	GGGGTTGGGGTTTTTTTTTTT <u>GCGATTT</u>
GCGA-Gwire2- do	SEQ ID NO:206	GGGGTTGGGGTTTTTTTTTTT <u>GCGATTTGCGATTT</u>
GCGA-Gwire2- tri	SEQ ID NO:207	GGGGTTGGGGTTTTTTTTTTT <u>GCGATTTGCGATTTGCGATTT</u>
ACGC-Gwire2	SEQ ID NO:184	GGGGTTGGGGTTTTTTTTTTT <u>ACGCTTT</u>
ACGC-Gwire2-	SEQ ID	GGGGTTGGGGTTTTTTTTTTT <u>ACGCTTTACGCTTT</u>

do	NO:208	
ACGC-Gwire2-tri	SEQ ID NO:209	GGGGTTGGGGTTTTTTTTTTTACGCTTTACGCTTTACGCTTT
TCGC-Gwire2	SEQ ID NO:187	GGGGTTGGGGTTTTTTTTTTTTCGCTTT
TCGC-Gwire2-do	SEQ ID NO:210	GGGGTTGGGGTTTTTTTTTTTTCGCTTTTCGCTTT
TCGC-Gwire2-tri	SEQ ID NO:211	GGGGTTGGGGTTTTTTTTTTTTCGCTTTTCGCTTTTCGCTTT
CCGC-Gwire2	SEQ ID NO:186	GGGGTTGGGGTTTTTTTTTTTCCGCTTT
CCGC-Gwire2-do	SEQ ID NO:212	GGGGTTGGGGTTTTTTTTTTTCCGCTTTCCGCTTT
CCGC-Gwire2-tri	SEQ ID NO:213	GGGGTTGGGGTTTTTTTTTTTCCGCTTTCCGCTTTCCGCTTT
GCGG-Gwire2-mo	SEQ ID NO:181	GGGGTTGGGGTTTTTTTTTTTGCGGTTT
GCGG-Gwire2-do	SEQ ID NO:214	GGGGTTGGGGTTTTTTTTTTTGCGGTTTGCGGTTT
GCGG-Gwire2-tri	SEQ ID NO:215	GGGGTTGGGGTTTTTTTTTTTGCGGTTTGCGGTTTGCGGTTT

Table 33: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
GCGT-Gwire2	2886	272
GCGT-Gwire2-do	44.5	426
GCGT-Gwire2-tri	13.7	457
GCGA-Gwire2	1996	220
GCGA-Gwire2-do	48.8	441
GCGA-Gwire2-tri	22.7	421
ACGC-Gwire2	3020	288
ACGC-Gwire2-do	379	267
ACGC-Gwire2-tri	46.0	441
TCGC-Gwire2	2232	341
TCGC-Gwire2-do	180	421
TCGC-Gwire2-tri	26.2	488
CCGC-Gwire2	3758	240
CCGC-Gwire2-do	74.2	401
CCGC-Gwire2-tri	4.4	428
GCGG-Gwire2	19903	22

GCGG-Gwire2-do	391	333
GCGG-Gwire2-tri	89.5	470

[0215] GCGT-Gwire2 showed the expected nanomolar EC₅₀. Addition of a second GCGTTTT element (GCGT-Gwire2-do, Table 32) at the 3'-end led to a massive improvement of EC₅₀ (by a factor of ~65) and an increase in V_{max} (Table 33, FIGs. 35A and 35B). Further addition of a GCGTTTT element (GCGT-Gwire2-tri, Table 32) led to another decrease in EC₅₀ by a factor of 3 (135 composite) and a slight increase in V_{max} (Table 33, FIGs. 35A and 35B).

[0216] GCGA-Gwire2 showed the expected nanomolar EC₅₀. Addition of a second GCGATTT element (GCGA-Gwire2-do, Table 32) at the 3'-end led to a strong improvement of EC₅₀ (by a factor of ~24) and an increase in V_{max} (Table 33, FIGs. 36A and 36B). Further addition of a GCGATTT element (GCGT-Gwire2-tri, Table 32) led to another decrease in EC₅₀ by a factor of 2 (48 composite) and a slight increase in V_{max} (Table 33 FIGs. 36A and 36B).

[0217] ACGC-Gwire2 showed the expected nanomolar EC₅₀. Addition of a second ACGCTTT element (ACGC-Gwire2-do, Table 32) at the 3'-end led to a mild improvement of EC₅₀ (by a factor of ~8) and an increase in V_{max} (Table 33, FIGs. 37A and 37B). Further addition of an ACGCTTT element (ACGC-Gwire2-tri, Table 32) led to another decrease in EC₅₀ by a factor of 8 (64 composite) and a slight increase in V_{max} (Table 33, FIGs. 37A and 37B).

[0218] TCGC-Gwire2 showed the expected nanomolar EC₅₀. Addition of a second TCGCTTT element (TCGC-Gwire2-do, Table 32) at the 3'-end led to a strong improvement of EC₅₀ (by a factor of ~12) and an increase in V_{max} (Table 33, FIGs. 38A and 38B). Further addition of a TCGCTTT element (TCGC-Gwire2-tri, Table 32) led to another decrease in EC₅₀ by a factor of 7 (84 composite) and a slight increase in V_{max} (Table 33, FIGs. 38A and 38B).

[0219] CCGC-Gwire2 showed the expected nanomolar EC₅₀. Addition of a second CCGCTTT element (CCGC-Gwire2-do, Table 32) at the 3'-end led to a massive improvement of EC₅₀ (by a factor of ~50) as well as V_{max} (Table 33, FIGs. 39A and 39B). Further addition of a CCGCTTT element (CCGC-Gwire2-tri, Table 32) led to another decrease in EC₅₀ by a factor of 17 (850 composite) and a slight increase in V_{max} (Table 33, FIGs. 39A and 39B).

[0220] GCGG-Gwire2 showed only weak signals in the low concentration range considered. Addition of a second GCGGTTTT element (GCGG-Gwire2-do, Table 32) at the 3'-end led to a massive improvement of EC₅₀ as well as V_{max} (by a factor of ~51) (Table 33, FIG. 40). Further addition of a GCGGTTTT element (GCGG-Gwire2-tri, Table 32) led to another decrease in EC₅₀ by a factor of 4 (204 composite) and a slight increase in V_{max} (Table 33 FIG. 40).

[0221] In summary, it is shown that addition of further CpG-containing TLR21-stimulatory sequence elements to oligonucleotides having a Gwire2 sequence and a single CpG element leads to EC₅₀ improvements from a factor of 8 to a factor of 55, while the V_{max} is typically doubled. Addition of a third element also uniformly improved TLR21 stimulatory activity further. It appears that this is a generic method to generate high activities from initial simple low activity hits.

Example 8: Achieving high activity TLR21-stimulatory ODNs with a synthesis/cost-of-goods advantage: addition of or nucleotide replacement by alkyl and ethylene glycol spacers a) between CpG-containing sequence elements, and b) within the G-quartet forming moiety and at its border to the CpG-containing sequence element

[0222] The 5'-Gwire2-technology (GGGGTTGGGGTTTT (SEQ ID NO:258)) was used to investigate the TLR21 stimulatory potency of a conceptually simple potential stimulatory sequence: three consecutive CpGs encased by four dTs at the 5'-end and three dTs at the 3'-end (Table 34 CG-Gw2-T0). The influence of spacing of the CpG elements on TLR21 stimulatory activity was investigated by stepwise insertion of one, two, three and four dTs between the three CpG elements (resulting in CG-Gw2-T1 - CG-Gw2-T4, Table 34). A TLR21 stimulation assay in HEK293-NFκB-bsd-cTLR21 cells to determine the ability of the ODNs in Table 34 to stimulate TLR21 as described in Example 3 was performed, and EC₅₀ and V_{max} values calculated (Table 35, FIGs. 41A and 41B).

Table 34: ODN sequences

CG-Gw2-T0	SEQ ID NO:216	GGGGTTGGGGTTTTTTTTTCGCGCGTTT
CG-Gw2-T1	SEQ ID NO:217	GGGGTTGGGGTTTTTTTTTCGTCGTCGTTT
CG-Gw2-T2	SEQ ID NO:218	GGGGTTGGGGTTTTTTTTTCGTTTCGTTTCGTTT
CG-Gw2-T3	SEQ ID NO:219	GGGGTTGGGGTTTTTTTTTCGTTTCGTTTCGTTT
CG-Gw2-T4	SEQ ID NO:220	GGGGTTGGGGTTTTTTTTTCGTTTCGTTTCGTTT

Table 35: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
CG-Gw2-T0	Inactive	-
CG-Gw2-T1	133	378
CG-Gw2-T2	617	350
CG-Gw2-T3	16.6	335
CG-Gw2-T4	11.3	333

[0223] Remarkably, CG-Gw2-T0 was completely inactive on TLR21 in the concentration range considered (up to 20 nM), while one spacing dT between the CpGs already led to a strongly stimulatory ODN with an EC₅₀ in the picomolar range. A second dT between the CpGs did not

improve activity, but dT3 and dT4 led to EC₅₀ of 16.6 and 11.3 pM, respectively (Table 35, FIGs. 41A and 41B). This suggests that the sheer presence of CpG elements is not enough for activity; they need to be in the right context.

Does TLR21 stimulatory activity require a base in the spacer group?

[0224] An ODN with deoxyribosephosphate bridges (“abasic sites”) between the CpGs, instead of dTs (CG-Gw2-abase) was synthesized. This ODN and the parental CG-Gw2-T1 were tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 36, FIGs. 42A and 42B).

Table 36: ODN sequences

ODN	SEQ ID NO	Sequence
CG-Gw2-T1	SEQ ID NO:217	GGGGTTGGGGTTTTTTTCGTCGTCGTTT
CG-Gw2-abase	SEQ ID NO:221	GGGGTTGGGGTTTTTTTCGXCGXCGTTT
X = abasic site		

Table 37: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
CG-Gw2-T1	133	378
CG-Gw2-abase	34	299

[0225] Surprisingly, CG-Gw2-abase (FIG. 43) showed even somewhat higher potency on TLR21 (EC₅₀ = 34 pM) than CG-Gw2-T1 (EC₅₀ = 133 pM), while the V_{max} was somewhat lower (Table 37, FIGs. 42A and 42B). This result shows that a base in the spacing nucleotide in the CG-Gw2-T1 ODN is not only not required for TLR21 stimulation, but has a negative impact on the EC₅₀.

Impact of linear spacer groups on TLR21 activity of CG-Gw2 ODNs

[0226] In this study, the dT nucleotide spacing two CpGs in CG-Gw2-T1 was replaced by either a “C18” hexaethyleneglycol linker, or a “C3” propanediol linker (Table 38). These ODNs were tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3

Table 38: ODN sequences

ODN	SEQ ID NO	Sequence
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CG-Gw2-T1	SEQ ID NO:217	GGGGTTGGGGTTTTTTTTTCGTCGTCGTTT
CG-Gwire2 = CG-Gw2-T3	SEQ ID NO:219	GGGGTTGGGGTTTTTTTTTCGTTTCGTTTCGTTT
CG-Gw2X1	SEQ ID NO:222*	GGGGTTGGGGTTTTTTTTTCGX1CGX1CGTTT
CG-Gw2X2	SEQ ID NO:223*	GGGGTTGGGGTTTTTTTTTCGX2CGX2CGTTT
X1=C18 X2=C3		
*As referred to herein, CG-Gw2X1 and CG-Gw2X2 refer to the full sequences shown in this table, including the X1 and X2 non-nucleotide linkers.		

Table 39: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
CG-Gw2-T1*	133*	378*
CG-Gwire2 = CG-Gw2-T3	12.5	183
CG-Gw2X1	inactive	-
CG-Gw2X2	96.5	284

*Taken from the previous study

[0227] While a C18 spacer, formed by hexaethyleneglycol when inserted between CpG elements of CG-Gw2 (Table 36, FIG. 43), leads to a TLR21-inactive ODN (Table 39, FIG. 44A), the same modification with a C3 spacer (1,3-propanediol, CG-Gw2X2, Table 38, FIG. 43) not only retains, but even slightly improves the efficacy of the parental CG-Gw2-T1 with respect to EC₅₀ (Table 39, FIG. 44B). Given the simplicity of the C3 spacer structure compared to a nucleotide (FIG. 43), this is a most remarkable result. Considering that an abasic site, like the C3 spacer, comprises three connected carbon atoms between the two phosphodiester bonds, it is possible that C3 is a simplified form of the highly active abasic site structure (see FIG. 43), and also efficiently supports activation of TLR21.

Investigations on the TLR21 stimulatory activity of G-wire and TGGGGT (SEQ ID NO: 265)- activated C3 spacer-connected CpG structures

[0228] In this study, either a GGGGTTGGGG (SEQ ID NO: 257) G-wire (CG-Gw2X2-1) or a TGGGGT (SEQ ID NO: 265) element (CG-G4T16X2-1) was connected to TTTTTTTTCG-X2-CGTTT (SEQ ID NO. 271) (Table 40), and the TLR21 stimulatory potency was assessed. Then, both ODNs were further modified by consecutive additions of a C3-spacers connected to a CpG motifs, yielding ODNs with three, four, and five CpG motifs, each separated by C3 (Table 40). Their activation potency on TLR21 was also assessed in HEK293-NFκB-bsd-cTLR21 cells as explained in Example 3 (Table 41, FIGs. 45 and 46).

Table 40: ODN sequences

ODN	SEQ ID NO	Sequence
CG-Gw2X2-1	SEQ ID NO:225*	GGGGTTGGGGTTTTTTTTTCGX2CGTTT
CG-Gw2X2-2	SEQ ID NO:223*	GGGGTTGGGGTTTTTTTTTCGX2CGX2CGTTT
CG-Gw2X2-3	SEQ ID NO:226*	GGGGTTGGGGTTTTTTTTTCGX2CGX2CGX2CGTTT
CG-Gw2X2-4	SEQ ID NO:227*	GGGGTTGGGGTTTTTTTTTCGX2CGX2CGX2CGX2CGTTT
CG-Gw2X2-5	SEQ ID NO:228*	GGGGTTGGGGTTTTTTTTTCGX2CGX2CGX2CGX2CGX2CGTTT
CG-G4T16X2-1	SEQ ID NO:229*	TGGGGTTTTTTTTTCGX2CGTTT
CG-G4T16X2-2	SEQ ID NO:230*	TGGGGTTTTTTTTTCGX2CGX2CGTTT
CG-G4T16X2-3	SEQ ID NO:231*	TGGGGTTTTTTTTTCGX2CGX2CGX2CGTTT
CG-G4T16X2-4	SEQ ID NO:232*	TGGGGTTTTTTTTTCGX2CGX2CGX2CGX2CGTTT
CG-G4T16X2-5	SEQ ID NO:233*	TGGGGTTTTTTTTTCGX2CGX2CGX2CGX2CGX2CGTTT
X2=C3		
*As referred to herein CG-Gw2X2-1 through -5 and CG-G4T16X2-1 through -5 refer to the full sequences shown in this table, including the X2 non-nucleotide linkers.		

Table 41: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
CG-Gw2X2-1	312	283
CG-Gw2X2-2	78.5	267
CG-Gw2X2-3	7.4	254
CG-Gw2X2-4	4.9	250
CG-Gw2X2-5	6.5	253
CG-G4T16X2-1	1408	266
CG-G4T16X2-2	21.6	259
CG-G4T16X2-3	5.1	297
CG-G4T16X2-4	5.6	279
CG-G4T16X2-5	5.7	283

[0229] In G-wire activation, the first ODN with two CpG motifs separated by C3 showed already picomolar activity ($EC_{50} = 312$ pM), albeit in the triple digit range. A third C3-separated CpG gave an EC_{50} of 78.5 pM, which compares well with the 96.5 pM determined for a second, separately synthesized batch (see Table 39). Addition of a fourth C3-separated CpG motif gave another 10-fold increase in potency ($EC_{50} = 7.4$ pM). Additions of fifth and sixth C3-separated CpG motifs retained the high potency and even resulted in minor improvement. These single digit picomolar potencies are amongst the highest activities seen so far on TLR21, a remarkable and unexpected feat for structural elements as simple as propanediolphosphate-separated CpG motifs (Table 41, FIGs. 44A and 44B). Replacing the G-wire element in the X2-1 to X2-5 series by the GTTTTG element known to promote parallel intermolecular G-quartet structures (Table 40) led to ODNs of similar, in part even superior potency (Table 41, FIGs. 46A and 46B).

Investigations of the impact of spacer length and detailed chemical structure on the TLR21 stimulatory activity of G-wire-activated C3 spacer-connected CpG motifs

[0230] In this study, a GGGGTTGGGG (SEQ ID NO: 257) G-wire was connected to TTTTTTTTCG-X-CGXCGTTT (SEQ ID NO:260) (Table 42), and the TLR21 stimulatory potency was assessed. X is a series of alkyldiol-phosphates used to separate CpG motifs (Table 42, FIG. 47), of which ODN-X3 was a repeat synthesis of CG-Gw2X2 (see Table 38) and CG-Gw2X2-2 (see Table 40). Furthermore, an oligonucleotide comprising an abasic spacer (Table 42, FIG. 49; see CG-Gw2-abase (SEQ ID NO:221 in Table 36)) as well as a triethyleneglycol derivative spacer (a “C8” linker, FIG. 49) were assayed for TLR21-stimulation in HEK293-NFκB-bsd-cTLR21 cells as described in Example 3.

Table 42: ODN sequences

ODN	SEQ ID NO	Sequence
CG-Gw2-T1	SEQ ID NO:217	GGGGTTGGGGTTTTTTTTTCGTCGTCGTTT
ODN-X2	SEQ ID NO:234*	GGGGTTGGGGTTTTTTTTTCGX2CGX2CGTTT (X2 = Ethanediol)
ODN-X3	SEQ ID NO:223*	GGGGTTGGGGTTTTTTTTTCGX3CGX3CGTTT (X3 = Propanediol)
ODN-X4	SEQ ID NO:235*	GGGGTTGGGGTTTTTTTTTCGX4CGX4CGTTT (X4 = Butanediol)
ODN-X6	SEQ ID NO:236*	GGGGTTGGGGTTTTTTTTTCGX6CGX6CGTTT (X6 = Hexanediol)
ODN-X9	SEQ ID NO:237*	GGGGTTGGGGTTTTTTTTTCGX9CGX9CGTTT (X9 = Nonanediol)

ODN-X12	SEQ ID NO:238*	GGGGTTGGGGTTTTTTTTTCGX12CGX12CGTTT (X12 = Dodecanediol)
ODN-Xab	SEQ ID NO:239	GGGGTTGGGGTTTTTTTTTCGXabCGXabCGTTT (Xab = dSpacer (abasic))
ODN-XtrEG	SEQ ID NO:240*	GGGGTTGGGGTTTTTTTTTCGXtrCGXtrCGTTT (Xtr = Triethyleneglycol)
*As referred to herein, ODN-X2, -X3, -X4, -X6, -X9, -X12, and -XtrEG refer to the full sequences shown in this table, including the X2, X3, X4, X6, X9, X12, and Xtr non-nucleotide linkers.		

Table 43: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
CG-Gw2-T1*	133*	378*
ODN-X2	368	566
ODN-X3	149	554
ODN-X4	91.6	522
ODN-X6	8176	680
ODN-X9	12644	372
ODN-X12	inactive	-
ODN-Xab	127	592
ODN-XtrEG	3095	427

*Taken from the previous study

[0231] Structurally, the spacing between the 3'-phosphate of one CpG element to the 5'-phosphate of the next CpG element in CG-Gw2-T1 is via three linked carbon atoms from 5'C to 4'C to 3'C (FIG. 49). The same distance is maintained, when an abasic site is used, as the lack of the base does not change the deoxyribose moiety. The very same arrangement is maintained in ODN-X3, where three methylene (-CH₂-) groups form the spacer between 3'- and 5'-phosphate groups (FIG. 47). Interestingly, their potency on TLR21, as determined by the EC₅₀, is highly similar (133 pM, 127 pM, and 149 pM, respectively; Table 43), suggesting that physical distance is more important than detailed chemical structure (e.g., presence of base, integrity of the deoxyribose moiety), since the simplest conceivable linker 1,3-propanediol partially maintaining the deoxyribose geometry does not seem to be a disadvantage (Table 43, FIGs. 49A and 49B).

[0232] Based on the finding that 1,3-propanediol is equivalent as a spacer to deoxythymine (dT) or an abasic site (Table 43), that was also already suggested by earlier experiments (Tables 36-39), we investigated the effect of spacer length on TLR21 activity.

[0233] The shorter derivative ethanediol (ODN-X2, Table 42, FIG. 47) was weaker in TLR21 stimulation activity compared to the 1,3-propanediol derivative ODN-X3 (Table 42, FIG. 47), by a factor of more than two (Table 43, FIGs. 50A and 50B). By contrast, spacer in the 1,4-

butanediol derivative ODN-X4 (Table 42, FIG. 47) conferred slightly higher activity (Table 43, FIGs. 50A and 50B), while further elongation by two additional methyl groups (1,6-hexanediol, ODN-X6) or 5 additional methylene groups (1,9-nonanediol, ODN-X9) (Table 42, FIG. 47) dramatically diminished the TLR21 stimulation potency by a factor of 89 and 138, respectively (Table 43, FIGs. 50A and 50B). A spacer with 12 methylene groups (1,12, dodecanediol, ODN-X12) (Table 42, FIG. 47) was completely inactive in the concentration range considered Table 43, FIGs. 50A and 50B). A triethyleneglycol (TEG) linker was also explored (ODN-XtrEG, Table 42, FIG. 48). This derivative corresponds sterically to a C8 linker. Therefore, it was remarkable that its TLR21 EC₅₀ was significantly lower than that of the 1,9-nonanediol derivative ODN-X9, and still lower than the EC₅₀ for the 1,6-hexanediol derivative ODN-X6) (see Table 43, FIG. 51)

Does the C3 spacer principle also function for CpG-containing tetranucleotide structures?

[0234] C3 spacer-(1,3-propanediol)-containing TLR21-active ODNs having CpG-containing tetranucleotide structures were examined. To this end, in a first experiment the 5'-G-wire sequence-containing ACGC-Gw2X1, ACGC-Gw2X2, CCGC-Gw2X1 and CCGC-Gw2X2 were synthesized and tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 44). This was followed by synthesis and TLR21 testing of the 5'-G4T16-containing ACGC-G4T16X2, and CCGC-G4T16X2 (Table 44).

Table 44: ODN sequences

ODN	SEQ ID NO	Sequence
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
*Hexaethyleneglycol		
**As referred to herein, ACGC-Gw2X1, CCGC-Gw2X1, ACGC-Gw2X2, CCGC-Gw2X2, ACGC-G4T16-X2, and CCGC-G4T16-X2 refer to the full sequences shown in this table, including the X1 and X2 non-		

nucleotide linkers.

Table 45: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
ACGC-Gw2X1	inactive	-
CCGC-Gw2X1	1238	64
ACGC-Gw2X2	112	307
CCGC-Gw2X2	91.3	323
ACGC-G4T16-X2	68.1	277
CCGC-G4T16-X2	20.3	230

[0235] C3 spacing of CpG tetranucleotide sequences is clearly capable of stimulating TLR21. ACGC-Gw2X2 and CCGC-Gw2X2 (Table 44) displayed TLR21 EC₅₀s (Table 45, FIGs. 52A and 52B) in a range observed previously for CG-Gw2X2/ODN-X3 (compare Tables 39 and 43). As observed previously for CG-Gw2X1 (Table 39), and as predicted by the structure-activity relationships, the hexaethyleneglycol (HEG, “C18”) derivatives ACGC-Gw2X1 and CCGC-Gw2X1 were inactive, or very weak, respectively (Table 45, FIGs. 52A and 52B). Replacement of the 5'-G-wire sequence by the other privileged 5'-structure identified by us earlier (TGGGGT (SEQ ID NO: 265), G4T16) yielded two derivatives, ACGC-G4T16-X2 and CCGC-G4T16-X2, with further improved EC₅₀ (Table 45, FIGs. 53A and 53B).

Impact of C3 and C18 linkers at/in the 5'-G-rich sequence of TLR21-activating ODNs

[0236] It was investigated if C3 spacers (1,3-propanediol) or C18 spacers (hexaethyleneglycol, HEG) can improve the activity of TLR21-active 5'-G-quartet-containing ODNs, when placed between the CpG motif and the G-quartet sequence, or when positioned within the G-quartet sequence. Two ODNs based on 2006-PDE5dG4 were synthesized. One with a C18 linker 3' of downstream the dG4 sequence (2006-PDE5dG4-X1) and one with a C3 linker at the same position (2006-PDE5dG4-X2) (Table 46). Furthermore 2006-G-wire1 was modified by replacing the T's in the GGGGTTGGGG (SEQ ID NO: 257) sequence by either a C18 linker (2006-5dG4-X3) or a C3 linker (2006-5dG4-X4). All these derivatives were tested in HEK293-NFκB-bsd-cTLR21 cells for their ability to stimulate TLR21 as described in Example 3 (Table 47).

Table 46: ODN sequences

ODN	SEQ ID NO	Sequence
2006-PDE5dG4	SEQ ID NO:17	GGGGTCGTCGTTTTGTCGTTTTGTCGTT
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-PDE-G-Wire1	SEQ ID NO:141	GGGGTTGGGGTCGTCGTTTTGTCGTTTTGTCGTT
*Hexaethyleneglycol **As referred to herein, 2006-PDE5dG4-X1 through -X4 refer to the full sequences shown in this table, including the X1, X2, X3, and X4 non-nucleotide linkers.		

Table 47: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
2006-PDE5dG4-N3	109	180
2006-PDE5dG4-X1	15.1	154
2006-PDE5dG4-X2	55.1	154
2006-PDE-G-Wire1	380	194
2006-PDE5dG4-X3	78.6	150
2006-PDE5dG4-X4	86.1	142

[0237] In 2006-PDE5dG4, the addition of the C18 spacer between dG4 and 2006-PDE improved TLR21 activity as measure by EC₅₀ more than 6-fold (Table 47, FIG. 54A). The C3 spacer at the same position improved TLR21 stimulation by a factor of 2 (Table 47, FIG. 54A). In 2006-PDE-G-wire1, the replacement of the two Ts in the G-wire sequence by the C18 spacer improved TLR21 activity as measure by EC₅₀ about 5-fold (Table 47, FIG. 54B). The C3 spacer at the same position improved TLR21 stimulation by a factor of 4 (Table 47, FIG. 54B). Taken together, the data suggests that the TLR21 activating properties are uncompromised by the presence of C18 or C3 linkers and that they lead to even more improved activities.

Example 9: 5'-cholesterol, but not 3'-cholesterol, modification of ODNs results in strongly increased TLR21 stimulatory activity

[0238] The impact of the classical 3'-cholesterol modification and the more rarely used 5'-cholesterol modification on TLR21 stimulatory potential of moderately and highly active ODN species was examined.

3'- cholesterol modification

[0239] A commonly applied 3'-cholesterol modification (FIG. 55) was applied to two highly TLR21-active ODNs, 2006-Gwire2 and 2006-T4-5dTG4T (Table 48). More specifically, ODNs comprising a 3' cholesterol moiety were purchased from Eurofins. The structure of the cholesterol moiety was based on 3' Cholesterol SynBase™ shown in [www_linktech_co_uk/products/modifiers/hydrophobic_group_cholesterol_palmitate_modification/969_3-cholesterol-synbase-cpg-1000-110](http://www.linktech.co.uk/products/modifiers/hydrophobic_group_cholesterol_palmitate_modification/969_3-cholesterol-synbase-cpg-1000-110). A TLR21 stimulation test as explained in Example 3 was performed.

Table 48: ODN sequences

ODN	SEQ ID NO	Sequence
2006-Gwire2	SEQ ID NO:142	GGGGTTGGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-Gw2-3C	SEQ ID NO:142	GGGGTTGGGGTTTTTCGTCGTTTTGTCGTTTTGTCGTTX 3'-Cholesteryl
2006-T4-5dTG4T	SEQ ID NO:251	TGGGGTTTTTTTCGTCGTTTTGTCGTTTTGTCGTT
2006-T4TG4T-3C	SEQ ID NO:251	TGGGGTTTTTTTCGTCGTTTTGTCGTTTTGTCGTTX 3'-Cholesteryl

Table 49: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
Measurement 1		
2006-Gwire2	19.7	389
2006-Gw2-3C	26.5	325
2006-T4-5dTG4T	19.2	372
2006-T4TG4T-3C	33.5	330
Measurement 2		
2006-Gwire2	13.7	291
2006-Gw2-3C	67.4	301
2006-T4-5dTG4T	11.7	298
2006-T4TG4T-3C	68.8	261

[0240] The results suggest that the TLR21-stimulatory activity of both ODNs did not improve upon 3'-cholesterol modification (Table 49, FIGs. 56A, 56B, 57A, 57B). The EC₅₀ of the 3'-cholesterol-modified ODNs even increase (Table 49), suggesting minor loss of TLR21 stimulatory activity.

[0241] ODNs comprising a 3' cholesterol moiety were purchased from Eurofins. The structure of the cholesterol moiety was based on 3' Cholesterol SynBase™ shown in

[www_linktech_co_uk/products/modifiers/hydrophobic_group_cholesterol_palmitate_modification/969_3-cholesterol-synbase-cpg-1000-110](http://www.linktech.co.uk/products/modifiers/hydrophobic_group_cholesterol_palmitate_modification/969_3-cholesterol-synbase-cpg-1000-110).

5'- cholesterol modification (I)

[0242] The much less commonly applied 5'-cholesterol modification (FIGs. 58A and 58B) was synthesized onto a highly TLR21-active ODN identified in the course of our studies, GCGT3-TG4T (Table 50). More specifically, ODNs comprising a 5' cholesterol moiety were ordered from Genelink, and the structure of the lipid moiety of these ODNs was based on the structure shown at [www_genelink_com/newsite/products/MODPDFFILES/26-6602.pdf](http://www.genelink.com/newsite/products/MODPDFFILES/26-6602.pdf). Other ODNs comprising a 5' cholesterol moiety were ordered from Sigma Aldrich. The structure of the lipid moiety of these ODNs was based the structures shown at [www_sigmaaldrich_com/content/dam/sigmaaldrich/docs/Sigma-Aldrich/General_Information/1/custom-oligonucleotide-modifications-guide.pdf](http://www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma-Aldrich/General_Information/1/custom-oligonucleotide-modifications-guide.pdf), pages 85/86. Other ODNs comprising a 5' cholesterol moiety were ordered from IBA Lifesciences and had a structure based on that shown at [www_iba-lifesciences_com/Services_custom_oligos_custom_DNA_Non-fluorescent_5-modifications.html](http://www.iba-lifesciences.com/Services_custom_oligos_custom_DNA_Non-fluorescent_5-modifications.html). A TLR21 stimulation test in HEK293-NFκB-bsd-cTLR21 cells as described in Example 3 was performed.

Table 50: ODN sequences (Upper case: PDE bonds, lower case PTO bonds)

ODN	SEQ ID NO	Sequence
5Chol-GCGT3-TG4T	SEQ ID NO:252	XTGGGGTTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT X = 5'-Cholesteryl
GCGT3-TG4T	SEQ ID NO:252	TGGGGTTTTTTTTTGCGTTTTTGCGTTTTTGCGTTTT
2006-PTO	SEQ ID NO:3	tcgtcgttttgcgttttgcgtt

Table 51: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
<i>Measurement 1</i>		
5Chol-GCGT3-TG4T	2.4	338
GCGT3-TG4T	352	356
2006-PTO	4479	427
<i>Measurement 2</i>		
5Chol-GCGT3-TG4T	4.1	338
GCGT3-TG4T	623	356
2006-PTO	8790	427

[0243] The results of two independent measurements suggest that the TLR21-stimulatory activity of GCGT3-TG4T is massively improved by 5'-cholesterol modification (Table 51, FIGs. 59A, 59B). Compared to its unmodified version, the EC₅₀ decreased more than two orders of magnitude in both assays (factors of 147 and 152, respectively, Table 51). The 5'-cholesteryl-modified GCGT3-TG4T is among the most active TLR21-stimulatory ODNs identified so far.

[0244] ODNs comprising a 5' cholesterol moiety were ordered from Genelink, and the structure of the lipid moiety of these ODNs was based on the structure shown at www.genelink.com/newsite/products/MODPDFFILES/26-6602.pdf. Other ODNs comprising a 5' cholesterol moiety were ordered from Sigma Aldrich. The structure of the lipid moiety of these ODNs was based the structures shown at www.sigmaaldrich.com/content/dam/sigmaaldrich/docs/Sigma-Aldrich/General_Information/1/custom-oligonucleotide-modifications-guide.pdf, pages 85/86. Other ODNs comprising a 5' cholesterol moiety were ordered from IBA Lifesciences and had a structure based on that shown at www.iba-lifesciences.com/Services_custom_oligos_custom_DNA_Non-fluorescent_5-modifications.html.

5'- cholesterol modification (I)

[0245] A 5'-cholesterol modification (FIGs. 58A and 58B) was synthesized onto two highly TLR21-active ODN identified in the course of our studies, GCGT-3-TG4T and GCGT-3-Gw2 (Table 52), and a TLR21 stimulation test in HEK293-NFκB-bd-cTLR21 cells as described in Example 3 was performed.

Table 52: ODN sequences (Sigma)

ODN	SEQ ID NO	Sequence
GCGT3-TG4T-5Chol	SEQ ID NO:252	XTGGGGTTTTTTTTTGC GTTTTTGCGTTTTTGCGTTTT 5'-Cholesteryl
GCGT3-TG4T	SEQ ID NO:252	TGGGGTTTTTTTTTGC GTTTTTGCGTTTTTGCGTTTT
GCGT3-Gw2-5Chol	SEQ ID NO:253	XGGGGTTGGGGTTTTTTTTTGC GTTTTTGCGTTTTTGCGTTTT 5'-Cholesteryl
GCGT-3-Gw2	SEQ ID NO:253	GGGGTTGGGGTTTTTTTTTGC GTTTTTGCGTTTTTGCGTTTT

Table 53: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
<i>Measurement 1 Titration from 20 nM</i>		

GCGT3-TG4T-5Chol	0.47	140
GCGT3-TG4T	4.5	134
GCGT-3-Gw2-5Chol	0.68	147
GCGT-3-Gw2	20.6	142
Measurement 2 Titration from 1 nM		
GCGT3-TG4T-5Chol	0.59	164
GCGT3-TG4T	7.5	159
GCGT-3-Gw2-5Chol	1.23	161
GCGT-3-Gw2	29.0	171

[0246] The results of two independent measurements suggest that the TLR21-stimulatory activity of both GCGT-3-TG4T and GCGT-3-Gw2 is massively improved by 5'-cholesterol modification (Table 53, FIGs. 60A, 60B, 61A, 61B). Compared to their unmodified versions, the EC₅₀ decreased by about 1 order of magnitude in both assays (factors of 10 and 13 for GCGT-3-TG4T-5Chol, and factors 30 and 24 for GCGT-3-Gw2-5Chol, respectively (Table 51). The 5'-cholesteryl-modified ODNs GCGT-3-TG4T and GCGT-3-Gw2 are the most active TLR21-stimulatory ODNs identified so far, exhibiting femtomolar EC₅₀ values.

5'- cholesterol modification (II)

[0247] A 5'-cholesterol modification (FIGs. 58A and 58B) was synthesized onto two highly TLR21-active ODN identified in the course of our studies, GCGT-3-TG4T and GCGT-3-Gw2 (Table 54), and a TLR21 stimulation test in HEK293-NFκB-bsd-cTLR21 cells as described in Example 3 was performed.

Table 54: ODN sequences (Sigma)

ODN	SEQ ID NO	Sequence
GCGT3-TG4T-5Chol	SEQ ID NO:252	XTGGGGTTTTTTTTTGC GTTTTTTGC GTTTTTTGC GTTTTT X = 5'-Cholesteryl
GCGT3-TG4T	SEQ ID NO:252	TGGGGTTTTTTTTTGC GTTTTTTGC GTTTTTTGC GTTTTT
GCGT3-Gw2-5Chol	SEQ ID NO:253	XGGGGTTGGGGTTTTTTTTTGC GTTTTTTGC GTTTTTTGC GTTTTT X = 5'-Cholesteryl
GCGT3-Gw2	SEQ ID NO:253	GGGGTTGGGGTTTTTTTTTGC GTTTTTTGC GTTTTTTGC GTTTTT
GCGT3-5Chol	SEQ ID NO:254	XTTTTTTTGC GTTTTTTGC GTTTTTTGC GTTTTT X = 5'-Cholesteryl
GCGT3	SEQ ID	TTTTTTTTGC GTTTTTTGC GTTTTTTGC GTTTTT

	NO:254	
2006-PTO	SEQ ID NO:3	tcgtcgttttgtcgttttgtcgtt

Table 55: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	Vmax milliOD 405nm/min (mOD405/min)
Measurement 1 Titration from 20 nM		
GCGT3-TG4T-5Chol	2.1	112
GCGT3-TG4T	11.5	94
GCGT3-Gw2-5Chol	2.3	94
GCGT3-Gw2	21.9	91
GCGT3- 5Chol	463	104
GCGT3	7840	99
2006-PTO	3931	114
Measurement 2 Titration from 1 nM		
GCGT3-TG4T-5Chol	3.4	119
GCGT3-TG4T	19.9	113
GCGT-3-Gw2-5Chol	5.2	115
GCGT-3-Gw2	53.6	120
GCGT3- 5Chol	665	145
GCGT3	weak	-

[0248] The results of two independent measurements suggest that the TLR21-stimulatory activity of both GCGT-3-TG4T and GCGT-3-Gw2 is improved by 5'-cholesterol modification (Table 55, FIGs. 62A, 62B, 63A, 63B). Compared to their unmodified versions, the EC₅₀ decreased about 5 to 10-fold in both assays (factor of approximately 5 for GCGT3-TG4T-5Chol and factor of approximately 10 for GCGT3-Gw2-5Chol (Table 55)). It was also shown in this study, that the 5'-dG sequences are not required for the activity-enhancing effect of 5'-cholesterol as GCGT3-5Chol is approximately 17-fold more active compared to its non-modified congener (Table 55, FIG. 64).

5'- cholesterol modification (III)

[0249] A 5'-cholesterol modification (FIGs. 58A and 58B) was synthesized onto GCGT3-TG4T by another supplier (Table 56), and a TLR21 stimulation test in HEK293-NFκB-bsd-cTLR21 cells as described in Example 3 was performed.

Table 56: ODN sequences (IBA GmbH)

ODN	SEQ ID NO	Sequence
GCGT3-	SEQ ID NO:252	XTGGGGTTTTTTTTTTCGTTTTTTCGTTTTTTCGTTTTT

TG4T-5Chol		X = 5'-Cholesteryl
GCGT3-TG4T	SEQ ID NO:252	TGGGGTTTTTTTTTCGCTTTTTCGCTTTTTCGCTTTT

Table 57: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
<i>Measurement 1 Titration from 20 nM</i>		
GCGT3-TG4T-5Chol	2.04	343
GCGT3-TG4T	2991	303
<i>Measurement 2 Titration from 1 nM</i>		
GCGT3-TG4T-5Chol	3.86	282
GCGT3-TG4T	weak	-

[0250] The 5'-cholesteryl-modified form of GCGT3-TG4T showed highly potent TLR21 stimulatory activity, with single digit pM EC₅₀ values (Table 57, FIGs. 65A and 65B). By contrast, the GCGT3-TG4T form devoid of 5'-cholesteryl was, with respect to EC₅₀, almost 1500-fold less potent, demonstrating the importance of the 5' lipid modification (Table 57, FIGs. 65A and 65B).

5'- cholesterol modification (IV)

[0251] A 5'-cholesterol modification (FIGs. 58A and 58B) was synthesized onto another highly TLR21-active ODN with a different CpG-tetranucleotide core identified in the course of our studies, CCGC3-Gw2 (Table 58), and a TLR21 stimulation test in HEK293-NFκB-bsd-cTLR21 cells as described in Example 3 was performed.

Table 58: ODN sequences (Sigma)

ODN	SEQ ID NO	Sequence
5Chol-CCGC3-Gw2	SEQ ID NO:255	XGGGGTTGGGGTTTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT X = 5'-Cholesteryl
CCGC3-Gw2	SEQ ID NO:255	GGGGTTGGGGTTTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT
5Chol-CCGC3	SEQ ID NO:256	XTTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT X = 5'-Cholesteryl
CCGC3	SEQ ID NO:256	TTTTTTTTCCGCTTTTCCGCTTTTCCGCTTT

Table 59: Half-maximum effective concentration (EC₅₀) and maximum signal velocity (V_{max})

ODN	EC ₅₀ picomolar (pM)	V _{max} milliOD 405nm/min (mOD405/min)
<i>Measurement 1 Titration from 20 nM</i>		

5Chol-CCGC3-Gw2	3.4	87
CCGC3-Gw2	19.4	83
5Chol-CCGC3	1564	146
CCGC3	51436	52
Measurement 2		
<i>Titration from 1 nM</i>		
5Chol-CCGC3-Gw2	8.4	107
CCGC3-Gw2	24.6	98
5Chol-CCGC3	weak	-
CCGC3	inactive	-

[0252] The results of two independent measurements suggest that the TLR21-stimulatory activity of CCGC3-Gw2 is improved by 5'-cholesterol modification (Table 59, FIGs. 66A, 66B). Compared to an unmodified version, the EC₅₀ decreased about 3 to 5-fold in both assays (Table 59). It was also shown in this study, that the 5'-dG sequences are not required for the activity-enhancing effect of 5'-cholesterol: CCGC3-5Chol is approximately 33-fold more active compared to its non-modified congener (Table 59, FIG. 67).

[0253] To summarize, this is believed to be the first report on increased intrinsic activity of ODNs on TLR21 due to a 5'-cholesteryl modification. This has been shown for GCGT3-TG4T (an ODN newly identified in this study series) in three different batches synthesized by three different suppliers. The TLR21 activity-increasing effect is in addition to the activation due to 5'-G-quartet-forming sequences (such as Gwire2 or TG4T), but does not require them (see GCGT3). The TLR21 activity-increasing effect has also been demonstrated for another CpG-ODN identified in this study series: CCGC3-Gwire2 and CCGC3. The 3'-cholesteryl modification does not have a TLR21 activity-increasing effect. It appears likely that a 5'-cholesteryl derivatization has also a stabilizing effect against nuclease degradation. It can be speculated that cholesteryl micelle assembly contributes to the formation of polydentate TLR21 ligands. The 5' location, as opposed to the 3' location, is likely to be required for correct orientation of the CpG motifs. Furthermore, it is possible that a 5'-cholesteryl derivatization has a modifying effect on bioavailability *in vivo*.

[0254] 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.

[0255] The disclosures of each patent, patent application, and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

Example 10: *In vivo* study of efficacy of immune stimulants in a Newcastle disease vaccination model in chickens

[0256] To determine the suitability and efficacy of ODN1, ODN2, and ODN3 as immune stimulants, each was tested in three different concentrations.

[0257] The following immune stimulants were investigated:

ODN1:[CholTEG]-TGGGGTTTTTTTTTTCGTTTTTTCGTTTTTTCGTTTTT
 (“GCGT3-TG4T-5Chol”) (SEQ ID NO:252) ([CholTEG]=5'-triethyleneglycol-linked
 cholesteryl modification),

ODN2:TGGGGTTTTTTTTTTCGTTTTTTCGTTTTTTCGTTTTT (“GCGT3-TG4T”)
 (SEQ ID NO:252),

ODN3:tcgtcgtttgcgttttgcgtt (“2006-PTO”) (SEQ ID NO:3).

[0258] Each immune stimulant was added to an oil emulsion containing a suboptimal concentration of an inactivated Newcastle disease virus (NDV) according to **Table 61**. For the preparation 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 60**).

[0259] The following parameters were tested: health of the chickens (data not shown) and serology by the Haemagglutination inhibition (HI) assay.

Table 60: Study Design

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

[0260] 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.

[0261] 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 61**).

Table 61: Preparation of Test Articles and Control Items

Group	Name	Water Phase				Oil Phase		
		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 ng in Stimune	-	2887	113	3	2	2.5	4.5
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: 252, see Table 50)		204		196		400	
ODN2	GCGT3-TG4T (SEQ ID NO: 252, see Table 50)		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	

[0262] 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.

[0263] 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.

[0264] Chickens allocated to group T12 were vaccinated with immune stimulant 1 (3 chickens), immune stimulant 2 (3 chickens) or immune stimulant 3 (3 chickens) in adjuvant (Stimune) at a ratio of 4:5. One chicken was vaccinated with dilution buffer in adjuvant (Stimune).

[0265] Chickens allocated to control groups of T13 (n=9) and T14 (n=9) were vaccinated with a suboptimal NDV suspension 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

[0266] 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 61**. 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.

[0267] General health was monitored by an experienced bio-technician daily from day of arrival until the end of the study.

Serum blood sampling

[0268] 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 °C.

Haemagglutination inhibition (HI) assay

[0269] 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.

[0270] 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

[0271] 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

[0272] No clinical symptoms or adverse events related to the vaccination were observed in any group. All chickens appeared healthy during the entire study period.

[0273] 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

[0274] The individual HI results expressed as Log2 titres of the 100 ng, 1000 ng and 5000 ng ODN1 dose groups are indicated in **Table 62**. The mean HI titres and standard deviation of these groups are indicated in **FIG. 70** (days 14 and 21 post vaccination (pv)) and **FIG. 71** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0275] 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 ($p=0.0243$).

[0276] 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. 70**), although the 1000 ng concentration is very close to significance.

Table 62:

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 SD			0.0 0.0			0.1 0.3				6.2 1.4				6.9 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 SD			0.0 0.0			0.1 0.3				6.9 1.1				7.3 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 SD			0.0 0.0			0.0 0.0				5.9 0.7				6.7 0.9	

ODN2, GCGT3-TG4T

[0277] The individual HI results expressed as Log₂ titres of the 100 ng, 1000 ng and 5000 ng ODN1 dose groups are indicated in **Table 63**. The mean HI titres and standard deviation of these groups are indicated in **FIG. 72** (days 14 and 21 pv) and **FIG. 73** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0278] The ODN2, GCGT3-TG4T 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 all three doses; 100 ng: mean HI titre 7.1 Log₂/SD 1.2 (p=0.0003), 1000 ng: mean HI titre 6.4 Log₂/SD 0.7 (p=0.0027) and 5000 ng: mean HI titre 6.1 Log₂/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 Log₂/SD 0.8 (p=0.0083) when compared to the NDV vaccine (HI titre 6.2 Log₂/SD 1.0). The mean HI titres for the 1000 ng and 5000 ng were 7.1 Log₂/0.6 (p=0.0696) and 7.2 Log₂/SD 1.0 (p=0.0956) respectively (**FIG. 72**).

Table 63:

T04	GCGT3-TG4T 100 ng	11462	0	0	0	0	0	7	6	7	6.7	7	7	8	7.3
		11464	0	0	0	0	0	8	7	8	7.7	7	8	8	7.7
		11466	0	0	0	0	0	7	6	6	6.3	8	8	7	7.7
		11468	0	0	0	0	0	8	7	8	7.7	8	9	8	8.3
		11470	0	0	0	0	0	7	6	7	6.7	7	7	7	7.0
		11472	0	0	0	0	0	10	10	9	9.7	10	9	8	9.0
		11474	0	0	0	0	0	7	6	6	6.3	7	7	7	7.0
		11476	0	0	0	0	0	6	5	5	5.3	7	7	6	6.7
		11478	0	0	0	0	0	8	6	6	6.7	7	7	7	7.0
		11480	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	6	6	6	6.0	7	7	7	7.0
		11484	0	0	0	0	0	6	6	7	6.3	7	7	7	7.0
		11486	0	0	0	0	0	6	6	6	6.0	7	7	7	7.0
		11488	0	0	0	0	0	6	8	6	6.7	8	8	8	8.0
		11490	0	0	0	0	0	5	5	5	5.0	6	6	6	6.0
		11492	0	0	0	0	0	7	7	7	7.0	7	7	8	7.3
		11494	0	0	0	0	0	7	7	7	7.0	7	7	7	7.0
		11496	0	0	0	0	0	6	6	6	6.0	7	8	7	7.3
		11498	0	0	0	0	0	8	7	7	7.3	9	7	8	8.0
		11500	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	8	7	7	7.3	10	8	9	9.0
		11504	0	0	0	0	0	7	7	6	6.7	8	7	7	7.3
		11506	0	0	0	0	0	7	6	6	6.3	7	6	7	6.7
		11508	0	0	0	0	0	6	5	5	5.3	8	6	7	7.0
		11510	0	0	0	0	0	8	7	7	7.3	9	8	8	8.3
		11512	0	0	0	0	0	8	6	7	7.0	9	7	8	8.0
		11514	0	0	0	0	0	5	5	5	5.0	6	6	7	6.3
		11516	0	0	0	0	0	7	6	6	6.3	7	7	7	7.0
		11518	0	0	0	0	0	6	5	5	5.3	7	6	8	7.0
		11520	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

[0279] 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 64**. 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 64**). Thus, for this animal and date the mean HI titre was based on the duplicate measurement.

[0280] The mean HI titres and standard deviation of these groups are indicated in **FIG. 74** (days 14 and 21 pv) and **FIG. 75** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0281] 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. 74).

Table 64:

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

Control groups

[0282] 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 65**. The mean HI titres and standard deviation of these groups are indicated in **FIG. 76** (days 14 and 21 pv) and **FIG. 77** (all data) compared to the mean titres of the diluted NDV vaccine group.

[0283] 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. 76**).

Table 65:

T10	Suboptimal vaccine (1:50)	11582	0	0	0	0	0	4	4	4	4.0	6	5	6	5.7
		11584	0	0	0	0	0	5	6	5	5.3	7	7	7	7.0
		11586	0	0	0	0	0	5	5	6	5.3	5	5	6	5.3
		11588	0	0	0	0	0	6	6	7	6.3	7	6	8	7.0
		11590	0	0	0	0	0	4	4	5	4.3	6	6	6	6.0
		11592	0	0	0	0	0	5	5	5	5.0	7	7	8	7.3
		11594	0	0	0	0	0	4	4	5	4.3	6	7	8	7.0
		11596	0	0	0	0	0	6	6	7	6.3	7	7	8	7.3
		11598	0	0	0	0	0	4	4	4	4.0	4	4	5	4.3
11600	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	8	8	8	8.0	9	9	10	9.3
		11604	0	0	0	0	0	9	9	8	8.7	8	9	10	9.0
		11606	0	0	0	0	0	7	7	8	7.3	8	8	9	8.3
		11608	0	0	0	0	0	8	9	9	8.7	9	9	10	9.3
		11610	0	0	0	0	0	9	9	9	9.0	10	9	10	9.7
		11612	0	0	0	0	0	8	8	9	8.3	8	8	8	8.0
		11614	0	0	0	0	0	9	8	9	8.7	8	7	8	7.7
		11616	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
11620	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	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
		11626	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
		11630	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
		11634	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
		11638	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		
		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	4	4	4	4.0	4	4	4	4.0
		11644	0	0	0	0	0	6	6	6	6.0	7	7	7	7.0
		11646	0	0	0	0	0	7	7	8	7.3	8	8	8	8.0
		11648	0	0	0	0	0	5	4	5	4.7	6	6	6	6.0
		11650	0	0	0	0	0	5	5	5	5.0	6	6	6	6.0
		11652	0	0	0	0	0	7	7	7	7.0	7	7	7	7.0
		11654	0	0	0	0	0	6	6	7	6.3	7	7	7	7.0
		11656	0	0	0	0	0	4	4	4	4.0	5	5	5	5.0
		11658	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	4	4	4	4.0	7	7	7	7.0
		11662	0	0	0	0	0	4	4	5	4.3	7	7	7	7.0
		11664	0	0	0	0	0	5	5	5	5.0	7	7	7	7.0
		11666	0	0	0	0	0	6	6	6	6.0	7	7	8	7.3
		11668	0	0	0	0	0	6	6	7	6.3	8	8	8	8.0
		11670	0	0	0	0	0	6	6	6	6.0	7	7	8	7.3
		11672	0	0	0	0	0	6	6	5	5.7	7	8	9	8.0
		11674	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
		mean			0.0			0.1			5.5			7.5	
		SD			0.0			0.2			0.8			0.4	

Conclusions

[0284] 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.

[0285] The following immune stimulants were investigated:

ODN1:[CholTEG]-TGGGGTTTTTTTTTTCGTTTTTTCGTTTTTTCGTTTTT (“GCGT3-TG4T-5Chol”) (SEQ ID NO:252) ([CholTEG]=5'-triethyleneglycol-linked cholesteryl modification),

ODN2:TGGGGTTTTTTTTTTCGTTTTTTCGTTTTTTCGTTTTT (“GCGT3-TG4T”) (SEQ ID NO:252),

ODN3: tcgctcggttttgcgcttttgcgcttttgcgcttt (“2006-PTO”) (SEQ ID NO:3).

[0286] 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.

[0287] 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.

[0288] 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.

[0289] 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.

[0290] 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.

[0291] 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.

[0292] 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.

[0293] 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.

[0294] 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.

[0295] 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.

[0296] 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. 78** and **FIG. 79**).

[0297] At day 21 pv no significant differences between all ODN groups were shown.

[0298] 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

[0299] For further illustration, additional non-limiting embodiments of the present disclosure are set forth below.

[0300] For example, embodiment 1 is an immunostimulatory oligonucleotide comprising at least one CpG motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

[0301] Embodiment 2 is the oligonucleotide of embodiment 1, wherein the guanine nucleotide enriched sequence comprises a first plurality of guanine nucleotides.

[0302] Embodiment 3 is the oligonucleotide of embodiment 2, wherein the first plurality of guanine nucleotides comprises three to eight guanine nucleotides.

[0303] Embodiment 4 is the oligonucleotide of embodiment 3, wherein 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, 143, or 252.

[0304] Embodiment 5 is the oligonucleotide of any one of embodiments 1 to 3, wherein the guanine nucleotide 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: 268), GGAGG, TGGAGGC, TGGAGGCTGGAGGC (SEQ ID NO:264), or TGGGGT (SEQ ID NO:265).

[0305] Embodiment 6 is the oligonucleotide of any one of embodiments 1 to 3, wherein 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.

[0306] Embodiment 7 is the oligonucleotide of any one of the preceding embodiments 1-6 further comprising a second plurality of guanine nucleotides between the first plurality of guanine nucleotides and the at least one CpG motif.

[0307] Embodiment 8 is the oligonucleotide of any one of embodiments 2 to 7, wherein the first plurality of guanine nucleotides, the second plurality of guanine nucleotides, or both comprise a G-quartet sequence.

[0308] Embodiment 9 is the oligonucleotide of embodiment 8, wherein the G-quartet sequence is an interaction site for other G-quartet sequences.

[0309] Embodiment 10 is the oligonucleotide of embodiment 9, wherein the G-quartet sequence comprises TGGGGT (SEQ ID NO: 265).

[0310] Embodiment 11 is the oligonucleotide of embodiment 7 wherein the first and second pluralities of guanine nucleotides comprise a G-wire sequence.

[0311] Embodiment 12 is the oligonucleotide of embodiment 11 the G-wire sequence is an interaction site for other G-wire sequences.

[0312] Embodiment 13 is the oligonucleotide of embodiment 10 or 11, wherein the G-wire sequence comprises SEQ ID NO:257 or 258.

[0313] Embodiment 14 is the oligonucleotide of embodiment 11 or 12, wherein the 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, 252, or GCGT-Gwire3.

[0314] Embodiment 15 is the oligonucleotide of any one of embodiments 7 to 14, wherein the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least one nucleotide.

[0315] Embodiment 16 is the oligonucleotide of any one of embodiment s 1 to 3, further comprising a linker between the first plurality of guanine nucleotides and the at least one CpG motif.

[0316] Embodiment 17 is the oligonucleotide of embodiment 16, wherein the linker comprises at least three nucleotides.

[0317] Embodiment 18 is the oligonucleotide of embodiment 16 or 17, wherein the linker comprises a hexaethyleneglycol, triethyleneglycol, propanediol, or derivatives thereof.

[0318] Embodiment 19 is the oligonucleotides of any one of embodiments 16 to 18, wherein the oligonucleotide comprises 2006-PDE5dG4-X1 or 2006-PDE5dG4-X3.

[0319] Embodiment 20 is the oligonucleotide of any one of the preceding embodiments 1-19, wherein the at least one CpG motif is a plurality of CpG motifs.

[0320] Embodiment 21 is the oligonucleotide of embodiment 20, wherein the plurality of CpG motifs comprises two, three, four, or five CpG motifs.

[0321] Embodiment 22 is the oligonucleotide of embodiment 20 or 21, wherein each CpG motif is separated from the other CpG motifs by at least one nucleotide or nucleotide analog.

[0322] Embodiment 23 is the oligonucleotide of embodiment 22, wherein the at least one nucleotide is one to four thymine nucleotides.

[0323] Embodiment 24 is the oligonucleotide of embodiment 22 or 23, wherein the oligonucleotide comprises SEQ ID NO:217, 218, 219, or 220.

[0324] Embodiment 25 is the oligonucleotide of embodiment 20, wherein each of the CpG motifs is separated from the other CpG motifs by a spacer.

[0325] Embodiment 26 is the oligonucleotide of embodiment 25, wherein the spacer is a deoxyribosephosphate bridge.

[0326] Embodiment 27 is the oligonucleotide of embodiment 26, wherein the deoxyribosephosphate bridge is abasic.

[0327] Embodiment 28 is the oligonucleotide of embodiment 27, wherein the oligonucleotide comprises SEQ ID NO:221.

[0328] Embodiment 29 is the oligonucleotide of embodiment 25, wherein the spacer comprises a carbon chain.

[0329] Embodiment 30 is the oligonucleotide of embodiment 29, wherein the carbon chain comprises two carbon atoms.

[0330] Embodiment 31 is the oligonucleotide of embodiment 30, wherein the carbon chain is derived from ethanediol.

[0331] Embodiment 32 is the oligonucleotide of embodiment 31, wherein the oligonucleotide comprises ODN-X2, wherein X2 is ethanediol.

[0332] Embodiment 33 is the oligonucleotide of embodiment 29, wherein the carbon chain comprises three carbon atoms.

[0333] Embodiment 34 is the oligonucleotide of embodiment 33, wherein the carbon chain is derived from 1,3-propanediol.

[0334] Embodiment 35 is the oligonucleotide of embodiment 33 or 34, wherein the nucleotide comprises CG-Gw2X2, 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 2006-PDE5dG4-X4, wherein X4 is a three carbon chain derived from propanediol.

[0335] Embodiment 36 is the oligonucleotide nucleotide of embodiment 29, wherein the carbon chain comprises four carbon atoms.

[0336] Embodiment 37 is the oligonucleotide of embodiment 36, wherein the carbon chain is derived from 1,4-butanediol.

[0337] Embodiment 38 is the oligonucleotide of embodiment 36 or 37, wherein the oligonucleotide comprises ODN-X4, wherein X4 is a four carbon chain derived from 1,4-butanediol.

[0338] Embodiment 39 is the oligonucleotide of embodiment 25, wherein the spacer comprises a repeated chemical unit.

[0339] Embodiment 40 is the oligonucleotide of embodiment 39, wherein the repeated chemical unit is an ethylene glycol.

[0340] Embodiment 41 is the oligonucleotide of embodiment 39 or 40, wherein the oligonucleotide comprises CCGC-Gw2X1, wherein X1 is a spacer derived from hexaethyleneglycol.

[0341] Embodiment 42 is the oligonucleotide of any one of the preceding embodiments 1-41 further comprising at least one nucleotide analog.

[0342] Embodiment 43 is the oligonucleotide of any one of the preceding embodiments 1-42 further comprising a phosphodiester backbone.

[0343] Embodiment 44 is the oligonucleotide of any one of the preceding embodiments 1-43 further comprising a phosphorothioate backbone.

[0344] Embodiment 45 is the oligonucleotide of any one of the preceding embodiments 1-44 further comprising a lipid moiety.

[0345] Embodiment 46 is the oligonucleotide of embodiment 45, wherein the lipid moiety is a cholesterol.

[0346] Embodiment 47 is the oligonucleotide of embodiment 45 or 46, wherein the lipid moiety is at or near the 5' terminus of the oligonucleotide.

[0347] Embodiment 48 is the oligonucleotides of any one of the preceding embodiments 1-47, wherein the CpG motif comprises a CpG sequence element having at least four nucleotides.

[0348] Embodiment 49 is the oligonucleotide of embodiment 48 comprising at least two CpG sequence elements.

[0349] Embodiment 50 is the oligonucleotide of embodiment 48 or 49 comprising at least three CpG sequence elements.

[0350] Embodiment 51 is the oligonucleotides of any one of embodiments 48 to 50, wherein the CpG sequence elements are GCGA, GCGG, ACGC, CCGC, GCGT, TCGC, or any combination thereof.

[0351] Embodiment 52 is the oligonucleotide of any one of the preceding embodiments 1-51 further comprising a tri-thymine nucleotide 3' terminal end.

[0352] Embodiment 53 is the oligonucleotide of embodiment 55, wherein the oligonucleotide comprises SEQ ID NO: 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, or 215.

[0353] Embodiment 54 is a vaccine for preventing or treating infectious disease comprising the oligonucleotide of any one of the preceding embodiments 1-53.

[0354] Embodiment 55 is a vector comprising the oligonucleotide of any one of the preceding embodiments 1-54.

[0355] Embodiment 56 is an immunostimulatory composition comprising the oligonucleotide of any one of the preceding embodiments 1-55.

[0356] Embodiment 57 is the immunostimulatory composition of embodiment 56 further comprising a pharmaceutically acceptable carrier.

[0357] Embodiment 58 is the immunostimulatory composition of embodiment 57, wherein the oligonucleotide and the carrier are linked.

[0358] Embodiment 59 is the immunostimulatory composition of embodiment 56 further comprising a hapten.

[0359] Embodiment 60 is the immunostimulatory composition of embodiment 57, wherein the oligonucleotide and the hapten are linked.

[0360] Embodiment 61 is a method of stimulating toll-like receptor 21 (TLR21) comprising:

administering to a subject in need thereof an immunostimulatory oligonucleotide having at least one CpG motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide, the guanine nucleotide enriched sequence comprising a first plurality of guanine nucleotides.

[0361] Embodiment 62 is the method of embodiment 61, wherein the concentration of the oligonucleotide is less than 20nM.

[0362] Embodiment 63 is the method of embodiment 61 or 62, wherein the oligonucleotide further comprises a pharmaceutically acceptable carrier.

[0363] Embodiment 64 is the method of any one of embodiments 61 to 63, wherein the immunostimulatory composition further comprises a hapten.

[0364] Embodiment 65 is the method of any one of embodiments 61 to 64, wherein the half maximum concentration (EC₅₀) of the oligonucleotide is less than 100 pM.

[0365] Embodiment 66 is the method of any one of embodiments 61 to 65, wherein the guanine nucleotide enriched nucleotide 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: 268), GGAGG, TGGAGGC, or TGGAGGCTGGAGGC (SEQ ID NO:264).

[0366] Embodiment 67 is the method of any one of embodiments 61 to 66, wherein the oligonucleotide comprises SEQ ID NO: 110, 111, 112, 113, 114, 115, 116, 117118, 119, 120, 124, 125, 126, 127, 129, 130, 131, 134, 136, 137, or 138.

[0367] Embodiment 68 is the method of any one of embodiments 61 to 67, wherein the oligonucleotide further comprises a G-wire sequence.

[0368] Embodiment 69 is the method of embodiment 68, wherein the G-wire sequence comprises SEQ ID NO:257 or 258.

[0369] Embodiment 70 is the method of embodiment 68, wherein the 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, 252, or GCGT-Gwire3.

[0370] Embodiment 71 is the method of any one of embodiments 61 to 70, comprising a second plurality of guanine nucleotides, wherein the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least one nucleotide.

[0371] Embodiment 72 is the method of any one of embodiments 61 to 71, wherein the oligonucleotide further comprises a linker between the first plurality of guanine nucleotides and the at least one CpG motif.

[0372] Embodiment 73 is the method of embodiment 72, wherein the linker comprises at least three nucleotides.

[0373] Embodiment 74 is the method of embodiment 72, wherein the linker comprises a hexaethyleneglycol, triethyleneglycol, propanediol, or derivatives thereof.

[0374] Embodiment 75 is the method of embodiment 72 to 74, wherein the oligonucleotide comprises 2006-PDE5dG4-X1 or 2006-PDE5dG4-X3.

[0375] Embodiment 76 is the method of embodiment 72 to 75, wherein the at least one CpG motif is a plurality of CpG motifs.

[0376] Embodiment 77 is the method of embodiment 76, wherein the plurality of CpG motifs comprises two, three, four, or five CpG motifs.

[0377] Embodiment 78 is the method of embodiment 76 or 77, wherein each CpG motif is separated from the other CpG motifs by at least one nucleotide or nucleotide analog.

[0378] Embodiment 79 is the method of embodiment 78, wherein the at least one nucleotide is one to four thymine nucleotides.

[0379] Embodiment 80 is the method of embodiment 78 or 79, wherein the oligonucleotide comprises SEQ ID NO:217, 218, 219, or 220.

[0380] Embodiment 81 is the method of embodiment 76 or 77, wherein each of the CpG motifs is separated from the other CpG motifs by a spacer.

[0381] Embodiment 82 is the method of embodiment 81, wherein the spacer is a deoxyribosephosphate bridge.

[0382] Embodiment 83 is the method of embodiment 82, wherein the deoxyribosephosphate bridge is abasic.

[0383] Embodiment 84 is the method of embodiments 82 or 83, wherein the oligonucleotide comprises SEQ ID NO:221.

[0384] Embodiment 85 is the method of embodiment 81, wherein the spacer comprises a carbon chain.

[0385] Embodiment 86 is the method of embodiment 85, wherein the carbon chain comprises two carbon atoms.

[0386] Embodiment 87 is the method of embodiment 86, wherein the carbon chain is derived from ethanediol.

[0387] Embodiment 88 is the method of embodiment 86 or 87, wherein the oligonucleotide comprises ODN-X2, wherein X2 is ethanediol.

[0388] Embodiment 89 is the method of embodiment 85, wherein the carbon chain comprises three carbon atoms.

[0389] Embodiment 90 is the method of embodiment 89, wherein the carbon chain is derived from 1,3-propanediol.

[0390] Embodiment 91 is the method of embodiment 89 or 90, wherein the nucleotide comprises CG-Gw2X2, 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 2006-PDE5dG4-X4, wherein X4 is a three carbon chain derived from propanediol.

[0391] Embodiment 92 is the method of embodiment 85, wherein the carbon chain comprises four carbon atoms.

[0392] Embodiment 93 is the method of embodiment 92, wherein the carbon chain is derived from 1,4-butanediol.

[0393] Embodiment 94 is the method of embodiment 92 or 93, wherein the oligonucleotide comprises ODN-X4, wherein X4 is a four carbon chain derived from 1,4-butanediol.

[0394] Embodiment 95 is the method of embodiment 81, wherein the spacer comprises a repeated chemical unit.

[0395] Embodiment 96 is the method of embodiment 95, wherein the repeated chemical unit is an ethylene glycol.

[0396] Embodiment 97 is the method of embodiment 95 or 96, wherein the oligonucleotide comprises CCGC-Gw2X1, wherein X1 is a spacer derived from hexaethyleneglycol.

[0397] Embodiment 98 is the method of any one of the preceding embodiments 61-97 further comprising at least one nucleotide analog.

[0398] Embodiment 99 is the method of any one of the preceding embodiments 61-98 further comprising a lipid moiety.

[0399] Embodiment 100 is the method of embodiment 99, wherein the lipid moiety is cholesterol.

[0400] Embodiment 101 is the method of embodiment 99 or 100, wherein the lipid moiety is at or near the 5' terminus of the oligonucleotide.

[0401] Embodiment 102 is the method of any one of embodiments 61 to 101, wherein the CpG motif comprises a CpG sequence element having at least four nucleotides.

[0402] Embodiment 103 is the method of embodiment 102, wherein the oligonucleotide comprises at least two CpG sequence elements.

[0403] Embodiment 104 is the method of embodiment 102 or 103, wherein the oligonucleotide comprises at least three CpG sequence elements.

[0404] Embodiment 105 is the method of any one of embodiments 101 to 104, wherein the CpG sequence elements are GCGA, GCGG, ACGC, CCGC, GCGT, TCGC, or any combination thereof.

[0405] Embodiment 106 is the method of embodiment 61 further comprising a tri-thymine nucleotide 3' terminal end.

[0406] Embodiment 107 is the method of embodiment 106, wherein the oligonucleotide comprises SEQ ID NO: 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, or 215.

[0407] Embodiment 108 is the method of any one of embodiments 61 to 107, wherein the immunostimulatory composition comprises a vaccine for preventing or treating infectious disease.

[0408] Embodiment 109 is the method of any one of embodiments 61 to 107, wherein the immunostimulatory composition comprises a vector.

[0409] Embodiment 110 is the method of any one of embodiments 61 to 109, wherein the immunostimulatory composition further comprises a pharmaceutically acceptable carrier.

[0410] Embodiment 111 is the method of embodiment 110, wherein the the oligonucleotide and the carrier are linked.

[0411] Embodiment 112 is the method of any one of embodiments 61 to 111, wherein the immunostimulatory composition further comprises a hapten.

[0412] Embodiment 113 is the method of any one of embodiments 112, wherein the oligonucleotide and the hapten are linked.

[0413] Embodiment 114 is the method of any one of embodiments 61 to 113, wherein the administering is performed intravenously, intramuscularly, intramammary, intradermally, intraperitoneally, subcutaneously, by spray, by aerosol, in ovo, mucosally, transdermally, by immersion, orally, intraocularly, intratracheally, or intranasally.

[0414] Embodiment 115 is the method of any one of embodiments 61 to 114, wherein the subject is an animal.

[0415] Embodiment 116 is the method of any one of embodiments 61 to 115, wherein the subject is a member of an avian species.

[0416] Embodiment 117 is a method for increasing TLR21-stimulatory activity of an oligonucleotide having at least one CpG motif comprising fusing the 5' end of the oligonucleotide to a guanine nucleotide enriched sequence.

[0417] Embodiment 118 is the method of embodiment 117, wherein the guanine nucleotide enriched sequence is a G-quartet sequence.

[0418] Embodiment 119 is the method of embodiment 118, wherein the G-quartet sequence comprises a first plurality of guanine nucleotides.

[0419] Embodiment 120 is the method of embodiment 119, wherein the first plurality of guanine nucleotides comprises three to eight guanine nucleotides.

[0420] Embodiment 121 is the method of embodiments 119 or 120, wherein the G-quartet 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: 268), GGAGG, TGGAGGC, or TGGAGGCTGGAGGC (SEQ ID NO:264).

[0421] Embodiment 122 is the method of any one of embodiments 117 to 121, wherein the oligonucleotide comprises a second plurality of guanine nucleotides.

[0422] Embodiment 123 is the method of any one of embodiments 117 to 122, wherein the guanine nucleotide enriched sequence comprises a G-wire sequence.

[0423] Embodiment 124 is the method of embodiment 123, wherein the G-wire sequence comprises SEQ ID NO:257 or 258.

[0424] Embodiment 125 is the method of any one of embodiments 119 to 124, wherein the first plurality of guanine nucleotides and the second plurality of guanine nucleotides are separated by at least one nucleotide.

[0425] Embodiment 126 is the method of embodiment 117, further comprising inserting a linker between the first plurality of guanine nucleotides and the at least one CpG motif.

[0426] Embodiment 127 is the method of embodiment 126, wherein the linker comprises at least three nucleotides.

[0427] Embodiment 128 is the method of embodiment 126 or 127, wherein the linker comprises a hexaethyleneglycol, triethyleneglycol, propanediol, or derivatives thereof.

[0428] Embodiment 129 is the method of any one of embodiments 126 to 128, wherein the oligonucleotide comprises 2006-PDE5dG4-X1 or 2006-PDE5dG4-X3.

[0429] Embodiment 130 is the method of embodiment 117, wherein the at least one CpG motif is a plurality of CpG motifs.

[0430] Embodiment 131 is the method of embodiment 130, wherein the plurality of CpG motifs comprises two, three, four, or five CpG motifs.

[0431] Embodiment 132 is the method of embodiment 130 or 131 further comprising inserting at least one nucleotide or nucleotide analog between the CpG motifs.

[0432] Embodiment 133 is the method of claim 132 wherein the at least one nucleotide is one to four thymine nucleotides.

[0433] Embodiment 134 is the method of embodiment 117, further comprising inserting a spacer between each of the CpG motifs.

[0434] Embodiment 135 is the method of embodiment 134, wherein the spacer is a deoxyribosephosphate bridge.

[0435] Embodiment 136 is the method of embodiment 135, wherein the deoxyribosephosphate bridge is abasic.

[0436] Embodiment 137 is the method of embodiment 134, wherein the spacer comprises a carbon chain.

[0437] Embodiment 138 is the method of embodiment 137, wherein the carbon chain comprises two carbon atoms.

[0438] Embodiment 139 is the method of embodiments 137 or 138, wherein the carbon chain is derived from ethanediol.

[0439] Embodiment 140 is the method of embodiment 137, wherein the carbon chain comprises three carbon atoms.

[0440] Embodiment 141 is the method of embodiment 137 or 140, wherein the carbon chain is derived from 1,3-propanediol.

[0441] Embodiment 142 is the method of embodiment 137, wherein the carbon chain comprises four carbon atoms.

[0442] Embodiment 143 is the method of embodiment 137 or 142, wherein the carbon chain is derived from 1,4-butanediol.

[0443] Embodiment 144 is the method of embodiment 137, wherein the spacer comprises a repeated chemical unit.

[0444] Embodiment 145 is the method of embodiment 137 or 144, wherein the repeated chemical unit is an ethylene glycol.

[0445] Embodiment 146 is the method of any one of embodiments 137, 144, or 145, wherein the spacer is derived from hexaethyleneglycol.

[0446] Embodiment 147 is the method of any one of embodiments 117 to 146 further comprising inserting at least one nucleotide analog.

[0447] Embodiment 148 is the method of any one of embodiments 117 to 147 further comprising inserting a lipid moiety.

[0448] Embodiment 149 is the method of embodiment 148, wherein the lipid moiety is a cholesterol.

[0449] Embodiment 150 is the method of embodiment 148 or 149, wherein the lipid moiety is at or near the 5' terminus of the oligonucleotide.

[0450] Embodiment 151 is the method of any one of embodiments 117 to 150, further comprising modifying the nucleotides adjacent to the CpG motif.

[0451] Embodiment 152 is a method of eliciting an immune response in a subject comprising:

administering to a subject in need thereof an immunostimulatory composition comprising an oligonucleotide having at least one CpG dinucleotide motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

[0452] Embodiment 153 is the method of embodiment 152, wherein the concentration of the oligonucleotide is less than 20nM.

[0453] Embodiment 154 is the method of embodiment 152 or 153, wherein the immunostimulatory composition further comprises a pharmaceutically acceptable carrier.

[0454] Embodiment 155 is the method of any one of embodiments 152 to 154, wherein the immunostimulatory composition further comprises a hapten.

[0455] Embodiment 156 is the method of any one of embodiments 152 to 155, wherein the half maximum concentration (EC₅₀) of the immunostimulatory composition is less than 100 pM.

[0456] Embodiment 157 is the method of embodiment 152, wherein the guanine nucleotide enriched sequence comprises a G-quartet sequence.

[0457] Embodiment 158 is the method of any one of embodiments 152 to 156, wherein the G-quartet 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: 268), GGAGG, TGGAGGC, TGGAGGCTGGAGGC (SEQ ID NO:264), or TGGGGT (SEQ ID NO:265).

[0458] Embodiment 159 is the method of any one of embodiments 152 to 157, wherein 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.

[0459] Embodiment 160 is the method of embodiment 152, wherein the guanine nucleotide enriched sequence comprises a G-wire sequence.

[0460] Embodiment 161 is the method of embodiment 160, wherein the G-wire sequence comprises SEQ ID NO:257 or 258.

[0461] Embodiment 162 is the method of embodiment 160, wherein the 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, 252, or GCGT-Gwire3.

[0462] Embodiment 163 is the method of embodiment 152, wherein the guanine nucleotide enriched sequence comprises first and second pluralities of guanine nucleotides separated by at least one nucleotide.

[0463] Embodiment 164 is the method of embodiment 152, wherein the oligonucleotide further comprises a linker between the guanine nucleotide enriched sequence and the at least one CpG motif.

[0464] Embodiment 165 is the method of embodiment 164, wherein the linker comprises at least three nucleotides.

[0465] Embodiment 166 is the method of embodiment 164, wherein the linker comprises a hexaethyleneglycol, triethyleneglycol, propanediol, or derivatives thereof.

[0466] Embodiment 167 is the method of embodiments 164 to 166, wherein the oligonucleotide comprises 2006-PDE5dG4-X1 or 2006-PDE5dG4-X3.

[0467] Embodiment 168 is the method of embodiment 152, wherein the at least one CpG motif is a plurality of CpG motifs.

[0468] Embodiment 169 is the method of embodiment 168, wherein the plurality of CpG motifs comprises two, three, four, or five CpG motifs.

[0469] Embodiment 170 is the method of embodiment 168 or 169, wherein each CpG motif is separated from the other CpG motifs by at least one nucleotide or nucleotide analog.

[0470] Embodiment 171 is the method of embodiment 170, wherein the at least one nucleotide analog is one to four thymine nucleotides.

[0471] Embodiment 172 is the method of embodiments 170 or 171, wherein the oligonucleotide comprises SEQ ID NO:217, 218, 219, or 220.

[0472] Embodiment 173 is the method of embodiment 168 or 169, wherein each of the CpG motifs is separated from the other CpG motifs by a spacer.

[0473] Embodiment 174 is the method of embodiment 173, wherein the spacer is a deoxyribosephosphate bridge.

[0474] Embodiment 175 is the method of embodiment 174, wherein the deoxyribosephosphate bridge is abasic.

[0475] Embodiment 176 is the method of embodiment 174 or 175, wherein the oligonucleotide comprises SEQ ID NO:221.

[0476] Embodiment 177 is the method of embodiment 173, wherein the spacer comprises a carbon chain.

[0477] Embodiment 178 is the method of embodiment 177, wherein the carbon chain comprises two carbon atoms.

[0478] Embodiment 179 is the method of embodiment 177 or 178, wherein the carbon chain is derived from ethanediol.

[0479] Embodiment 180 is the method of any one of embodiments 177 to 179, wherein the oligonucleotide comprises ODN-X2 wherein X2 is ethandiol.

[0480] Embodiment 181 is the method of embodiment 177, wherein the carbon chain comprises three carbon atoms.

[0481] Embodiment 182 is the method of embodiment 177 or 181, wherein the carbon chain is derived from 1,3-propanediol.

[0482] Embodiment 183 is the method of any one of embodiments 177, 181, or 182, wherein the nucleotide comprises CG-Gw2X2, 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 2006-PDE5dG4-X4, wherein X4 is a three carbon chain derived from propanediol.

[0483] Embodiment 184 is the method of embodiment 177, wherein the carbon chain comprises four carbon atoms.

[0484] Embodiment 185 is the method of embodiment 177 or 184, wherein the carbon chain is derived from 1,4-butanediol.

[0485] Embodiment 186 is the method of any one of embodiments 177, 184, or 185 wherein the oligonucleotide comprises ODN-X4, wherein X4 is a four carbon chain derived from 1,4-butanediol.

[0486] Embodiment 187 is the method of embodiment 173, wherein the spacer comprises a repeated chemical unit.

[0487] Embodiment 188 is the method of embodiment 187, wherein the repeated chemical unit is an ethylene glycol.

[0488] Embodiment 189 is the method of embodiments 187 or 188, wherein the oligonucleotide comprises CCGC-Gw2X1 and wherein X1 is a spacer derived from hexaethyleneglycol.

[0489] Embodiment 190 is the method of any one of embodiments 152 to 189 further comprising at least one nucleotide analog.

[0490] Embodiment 191 is the method of embodiments 152 to 190 further comprising attaching a lipid moiety into the oligonucleotide.

[0491] Embodiment 192 is the method of embodiment 191, wherein the lipid moiety is cholesterol.

[0492] Embodiment 193 is the method of embodiment 191 or 192, wherein the lipid moiety is at or near the 5' terminus of the oligonucleotide.

[0493] Embodiment 194 is the method of embodiment 152, wherein the CpG motif comprises a CpG sequence element having at least four nucleotides.

[0494] Embodiment 195 is the method of embodiment 194 comprising at least two CpG sequence elements.

[0495] Embodiment 196 is the method of embodiment 194 or 195 comprising at least three CpG sequence elements.

[0496] Embodiment 197 is the method of any one of embodiments 194 to 196, wherein the CpG sequence elements are GCGA, GCGG, ACGC, CCGC, GCGT, TCGC, or any combination thereof.

[0497] Embodiment 198 is the method of embodiment 152 further comprising inserting a tri-thymine nucleotide run onto the 3' terminal end of the oligonucleotide.

[0498] Embodiment 199 is the method of embodiment 198, wherein the oligonucleotide comprises SEQ ID NO: 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, or 215.

[0499] Embodiment 200 is an immunostimulatory oligonucleotide comprising SEQ ID NO:252.

[0500] Embodiment 201 is the oligonucleotide of embodiment 200, further comprising a 5' cholesteryl modification.

[0501] Embodiment 202 is the oligonucleotide of embodiment 201, wherein the 5' cholesteryl modification comprises a triethyleneglycol linker.

[0502] 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.

[0503] In view of the above, it will be seen that the several objects of the disclosure are achieved and other advantageous results attained.

[0504] 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.

[0505] What is claimed:

1. An immunostimulatory oligonucleotide comprising at least one CpG motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.
2. The oligonucleotide of claim 1, wherein the guanine nucleotide enriched sequence comprises a first plurality of guanine nucleotides.
3. The oligonucleotide of claim 2, wherein the first plurality of guanine nucleotides comprises three to eight guanine nucleotides.
4. The oligonucleotide of claim 3, wherein 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, 143, or 252.
5. The oligonucleotide of any one of claims 1 to 3, wherein the guanine nucleotide 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: 268), GGAGG, TGGAGGC, TGGAGGCTGGAGGC (SEQ ID NO:264), or TGGGGT (SEQ ID NO:265).
6. The oligonucleotide of any one of claims 1 to 3, wherein 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.
7. The oligonucleotide of any one of the preceding claims further comprising a second plurality of guanine nucleotides between the first plurality of guanine nucleotides and the at least one CpG motif.
8. The oligonucleotide of any one of claims 2 to 7, wherein the first plurality of guanine nucleotides, the second plurality of guanine nucleotides, or both comprise a G-quartet sequence.

9. The oligonucleotide of claim 7 wherein the first and second pluralities of guanine nucleotides comprise a G-wire sequence.
10. A vaccine for preventing or treating infectious disease comprising the oligonucleotide of any one of the preceding claims.
11. A vector comprising the oligonucleotide of any one of the preceding claims.
12. An immunostimulatory composition comprising the oligonucleotide of any one of the preceding claims.
13. The immunostimulatory composition of claim 12 further comprising a pharmaceutically acceptable carrier.
14. The immunostimulatory composition of claim 13, wherein the oligonucleotide and the carrier are linked.
15. A method of stimulating toll-like receptor 21 (TLR21) comprising:
 - a. administering to a subject in need thereof an immunostimulatory oligonucleotide having at least one CpG motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide, the guanine nucleotide enriched sequence comprising a first plurality of guanine nucleotides.
16. The method of claim 15, wherein 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.
17. The method of claim 16, wherein the oligonucleotide further comprises a G-wire sequence.
18. The method of claim 17, wherein the 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, 252, or GCGT-Gwire3.

19. A method for increasing TLR21-stimulatory activity of an oligonucleotide having at least one CpG motif comprising fusing the 5' end of the oligonucleotide to a guanine nucleotide enriched sequence.

20. A method of eliciting an immune response in a subject comprising:

- a. administering to a subject in need thereof an immunostimulatory composition comprising an oligonucleotide having at least one CpG dinucleotide motif and a guanine nucleotide enriched sequence beginning at or within four nucleotides of the 5' terminus of the oligonucleotide.

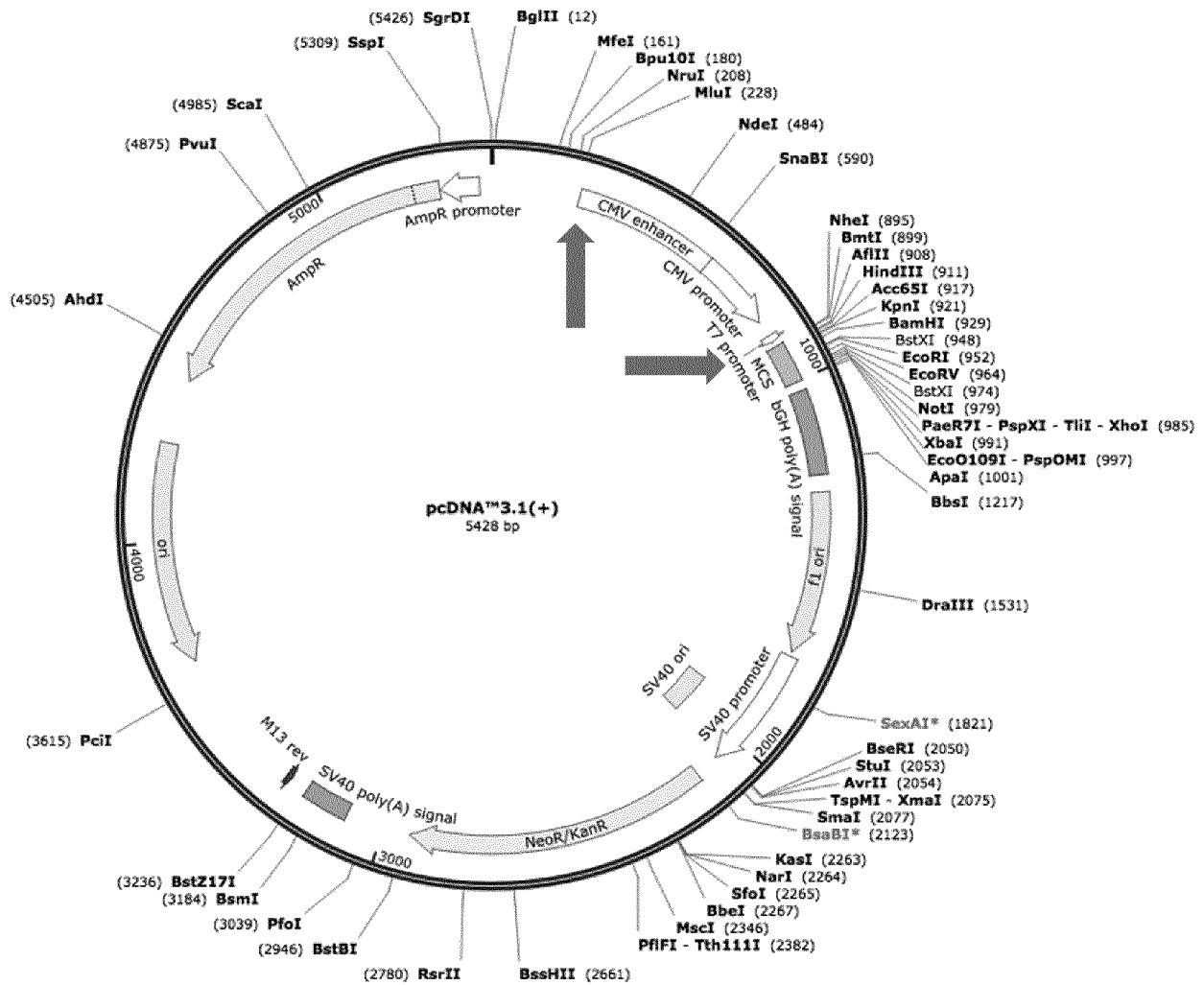


FIG. 1

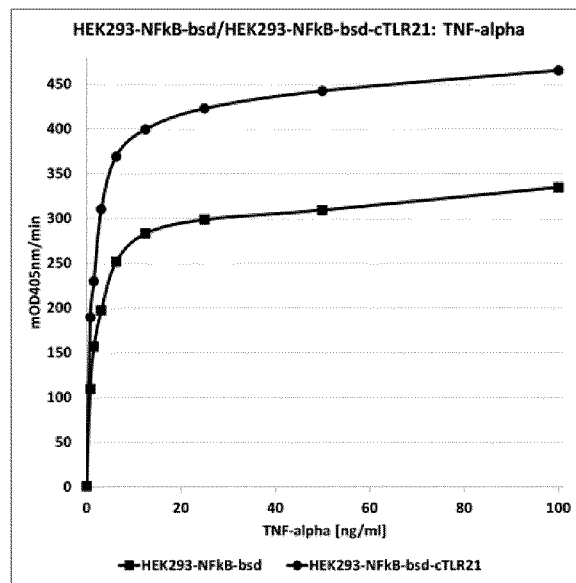


FIG. 2

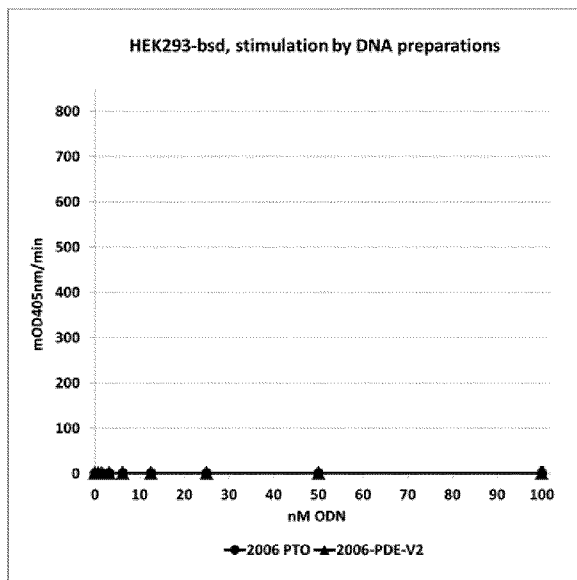


FIG. 3A

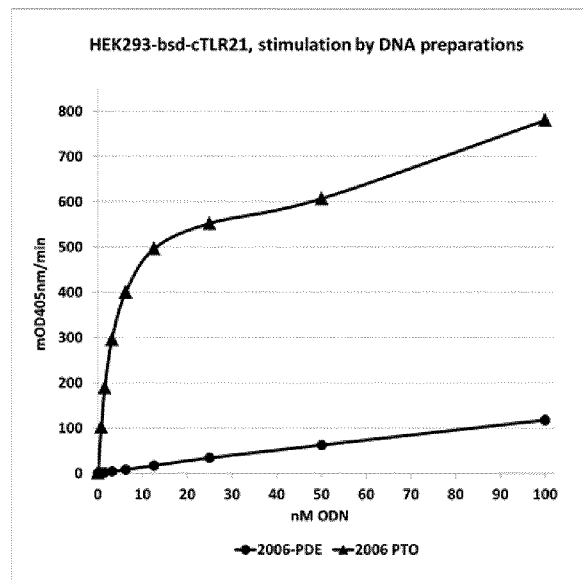


FIG. 3B

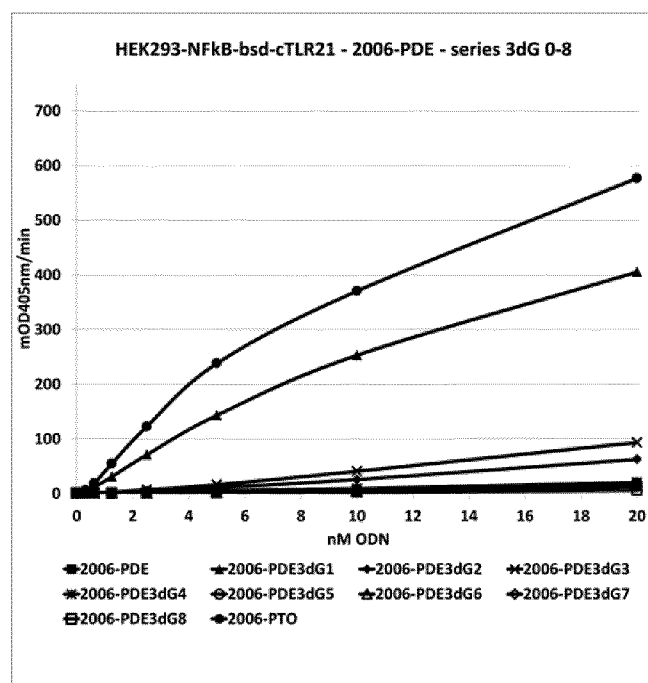


FIG. 4A

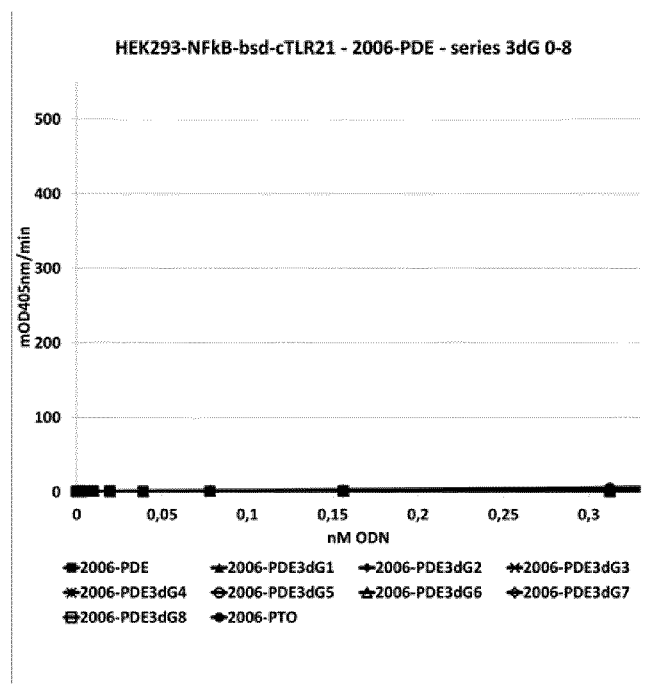


FIG. 4B

(TLR21, Titration of 2006-PDE-3dG0-8)

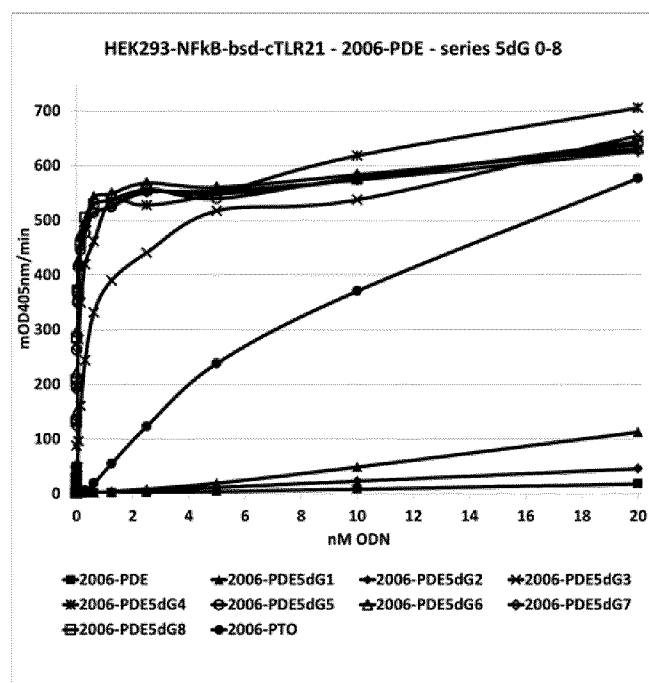


FIG. 5A

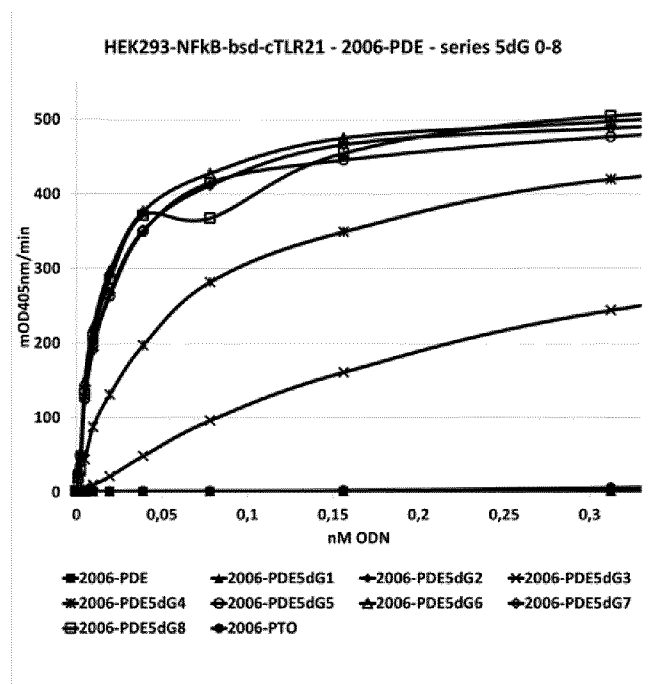
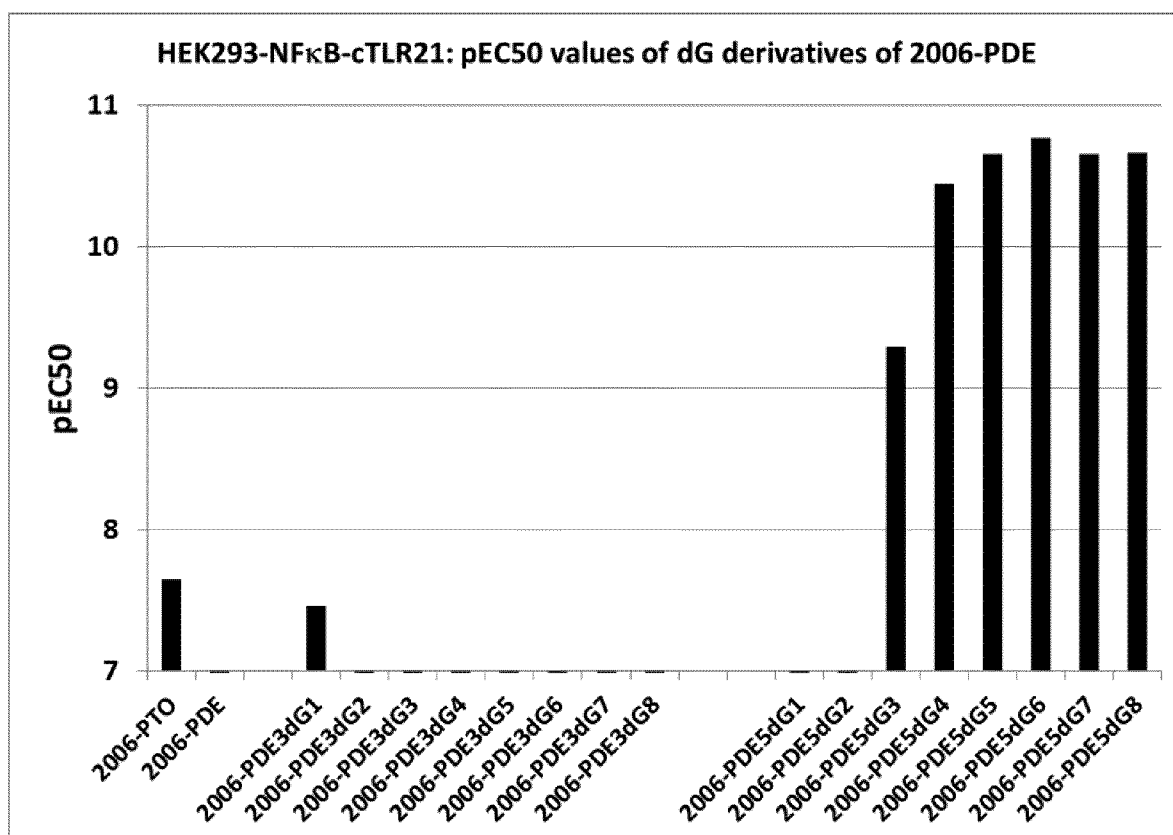
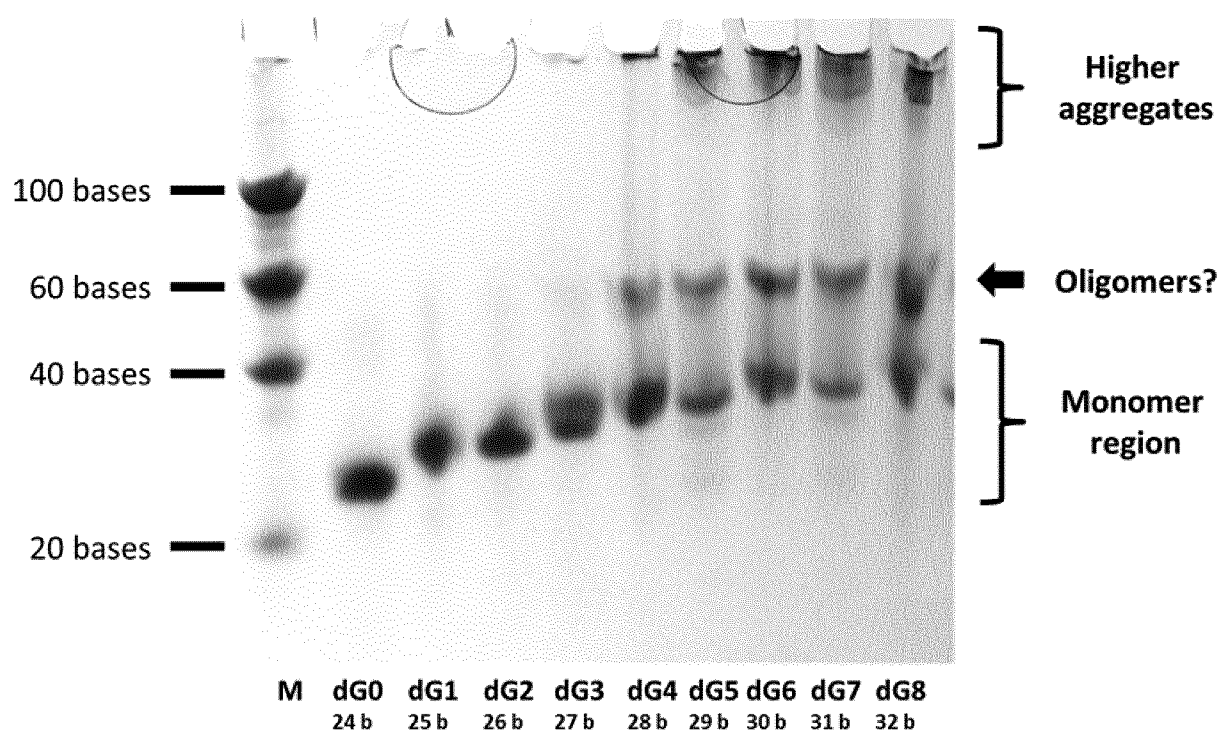


FIG. 5B

(TLR21, Titration of 2006-PDE-5dG0-8 stimulatory effects)

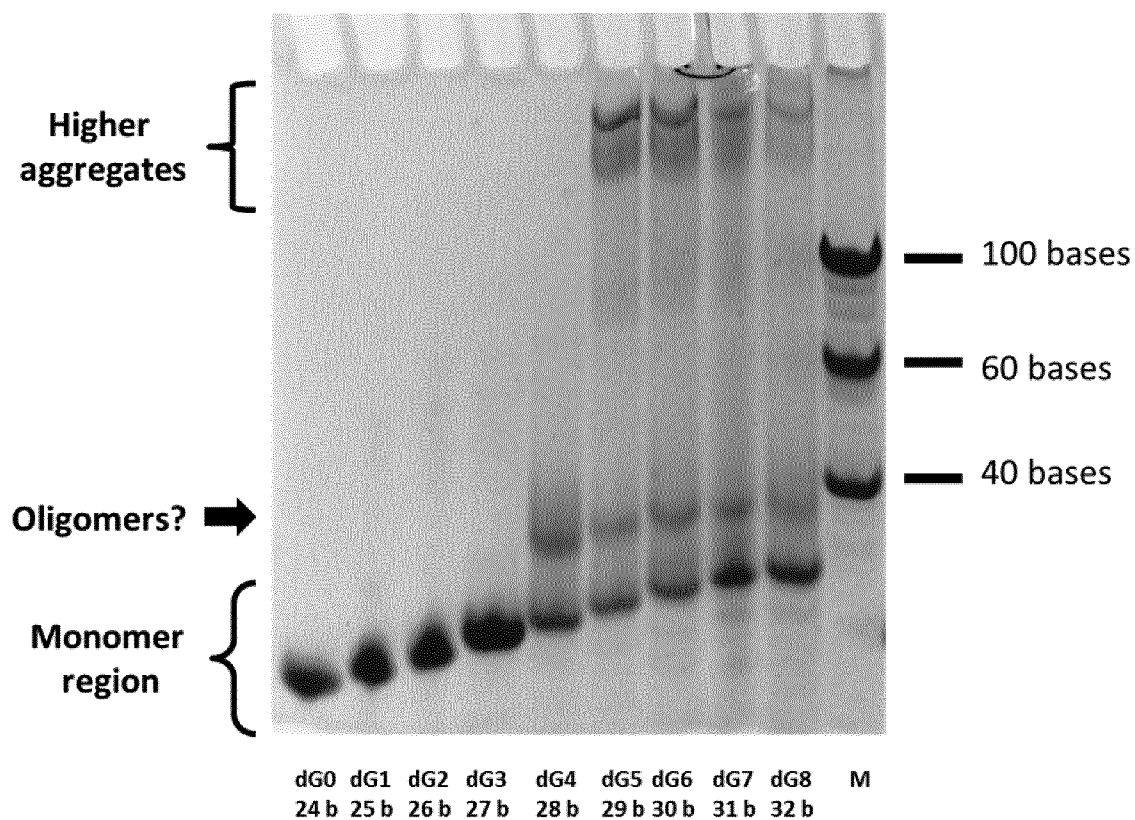
**FIG. 6**

(TLR21, 2006-PTO, 2006-PDE-3dG0-8 and 2006-PDE-5dG0-8 pEC50 values of TLR21 stimulatory activity)



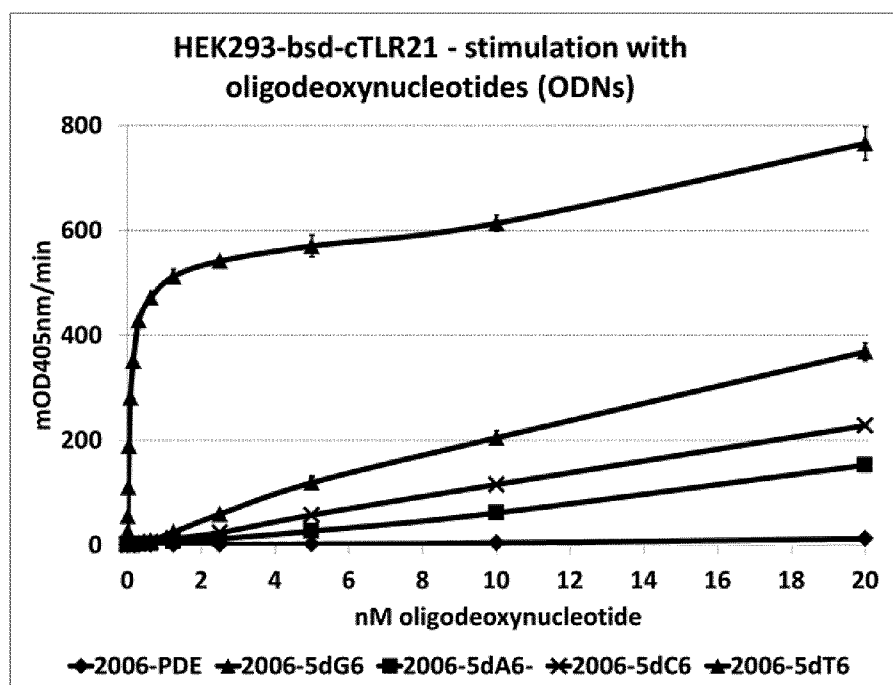
M: Mixture of 20, 40, 60 and 100 bases long ODNs; *dG0* = 2006-PDE; *dG1*-*dG8* corresponds to the 5'-dG-elongated 2006-PDE species 2006-PDE-5dG1 – 2006-PDE-5dG8. The respective ODN lengths in bases (b) is indicated. Native 16% TBE polyacrylamide gel electrophoresis, methylene blue staining.

FIG. 7A



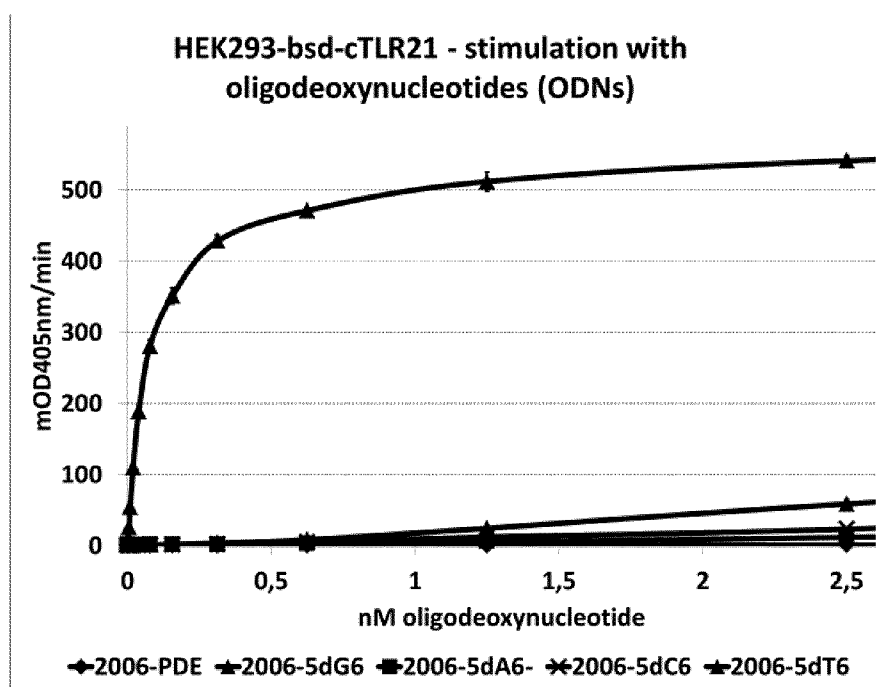
Mixture of 40, 60 and 100 bases long ODNs; dG0 = 2006-PDE; dG1-dG8 corresponds to the 3'-dG-elongated 2006-PDE species 2006-PDE-3dG1 – 2006-PDE-3dG8. The respective ODN lengths in bases (b) is indicated. Native 16% TBE polyacrylamide gel electrophoresis, methylene blue staining.

FIG. 7B



Titration down from 20 nM.

FIG. 8A



Expanded view of the titration range up to 2.5 nM.

FIG. 8B

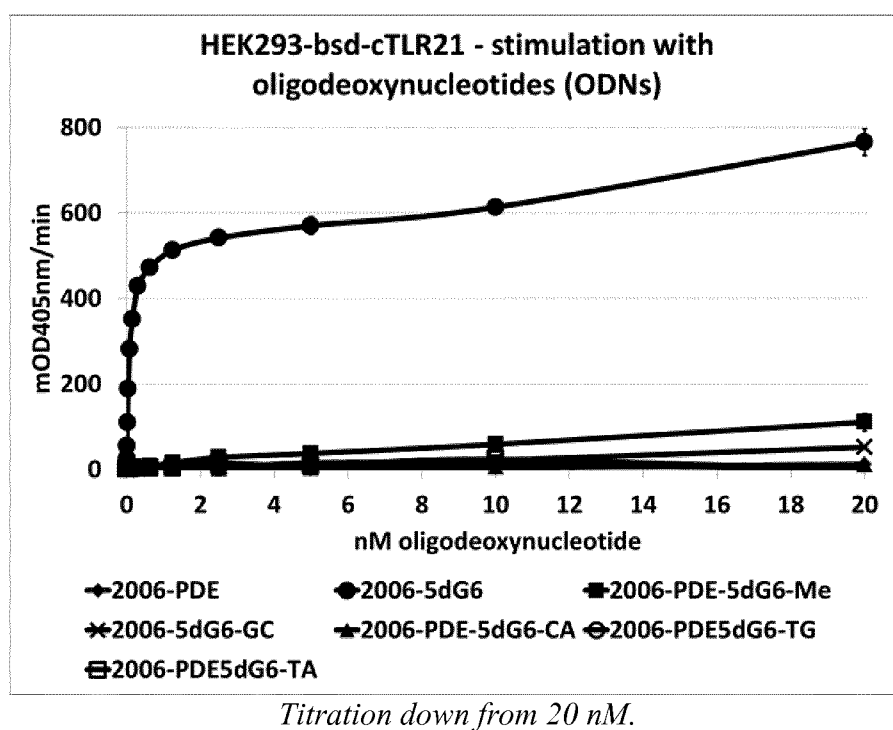


FIG. 9A

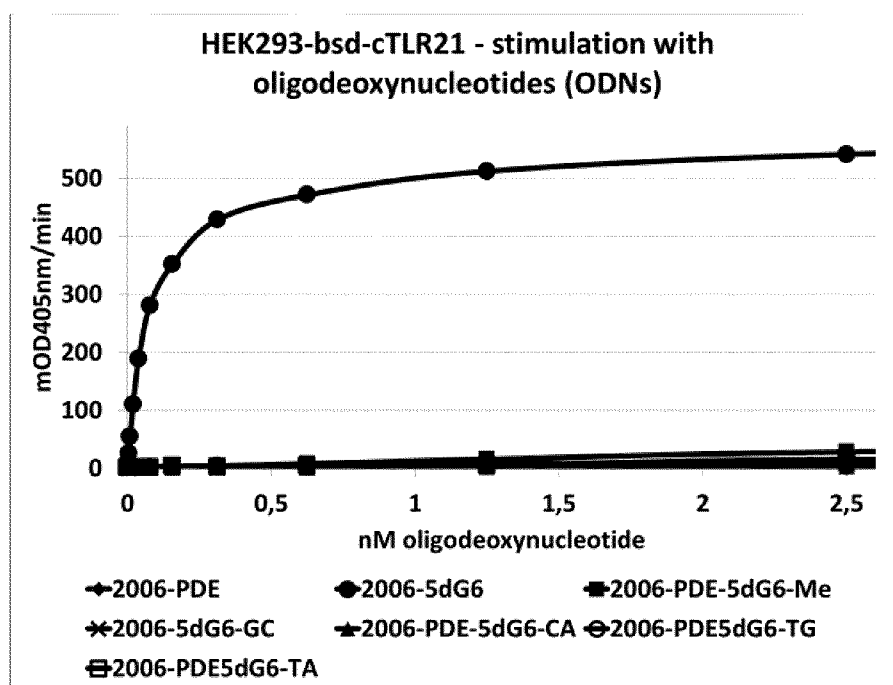


FIG. 9B

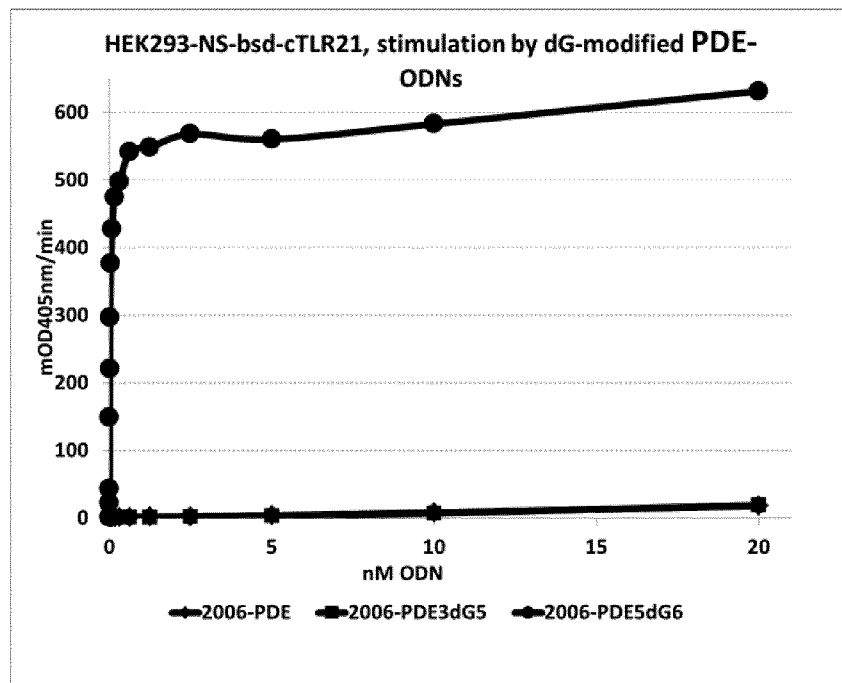


FIG. 10A

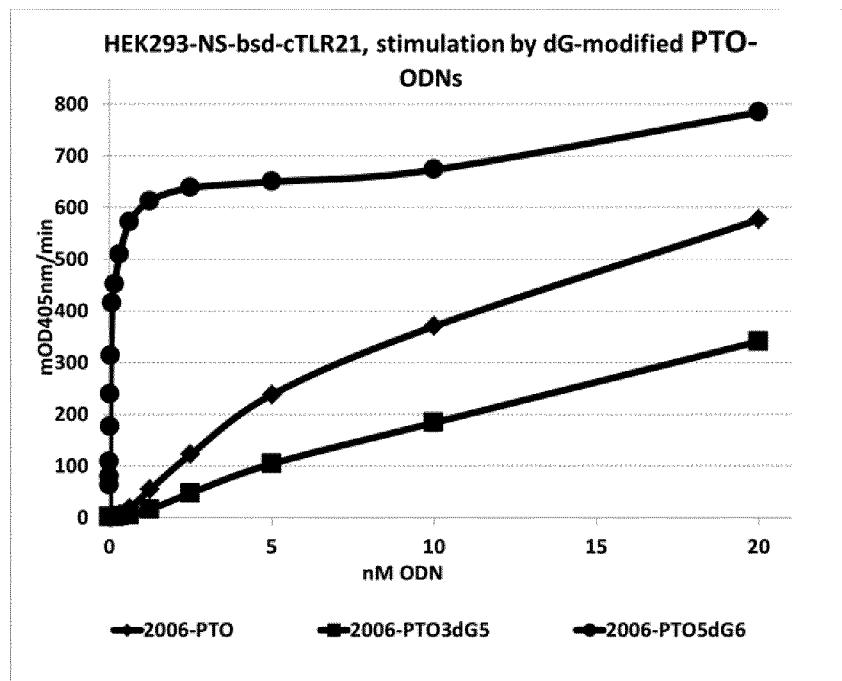


FIG. 10B

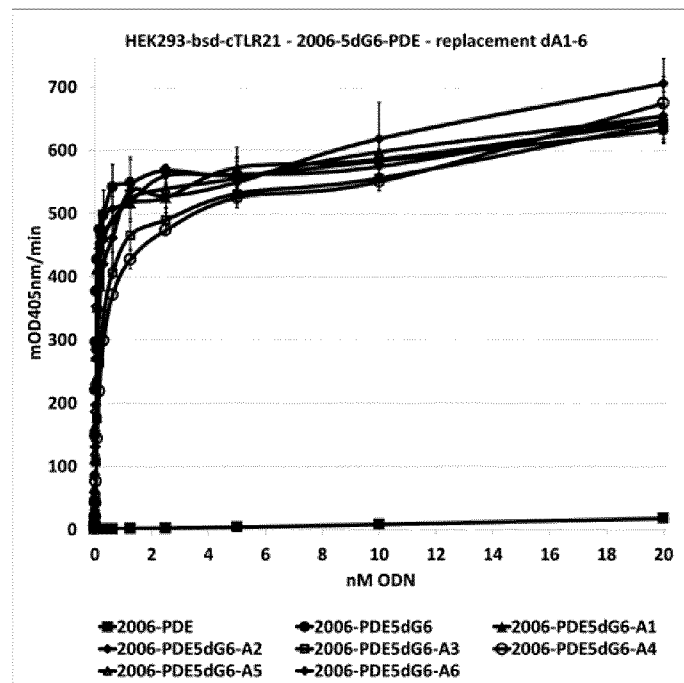


FIG. 11A

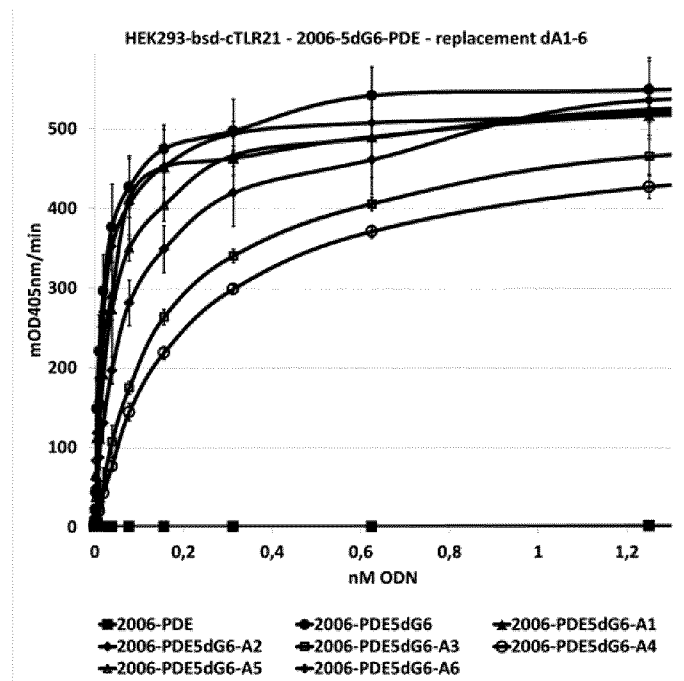


FIG. 11B

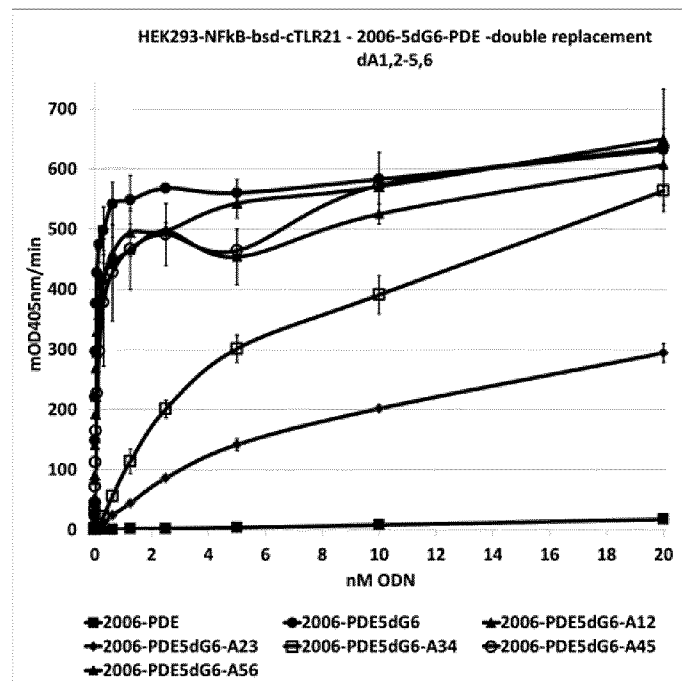


FIG. 11C

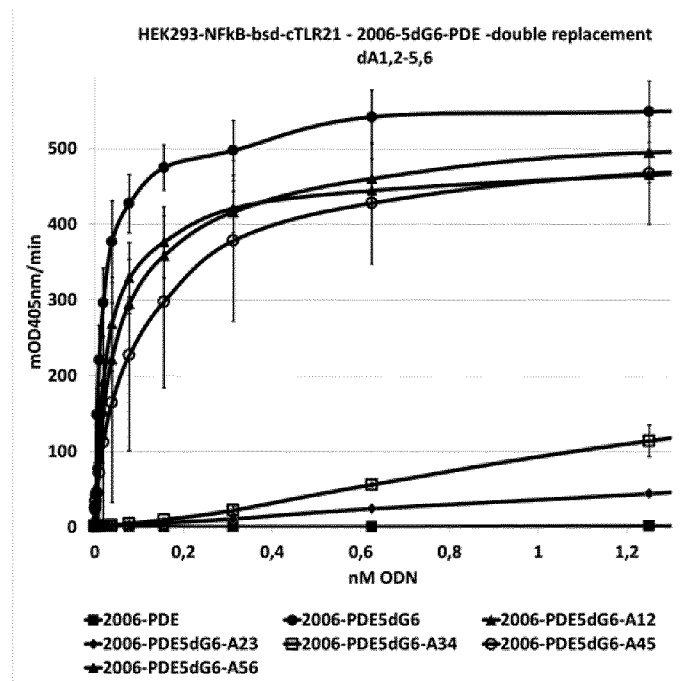


FIG. 11D

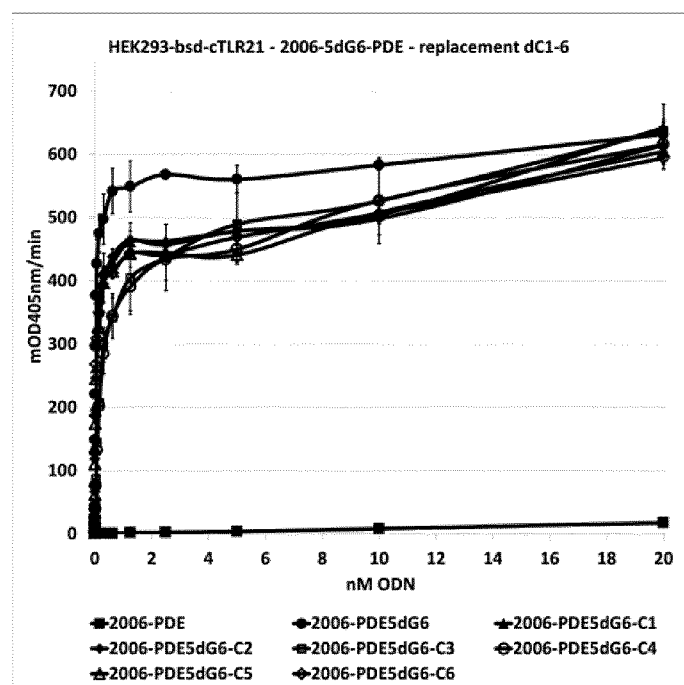


FIG. 12A

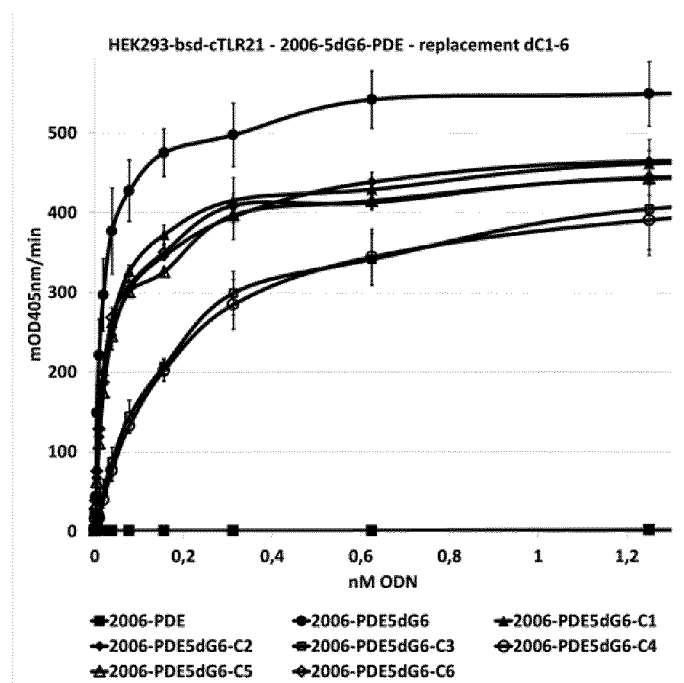


FIG. 12B

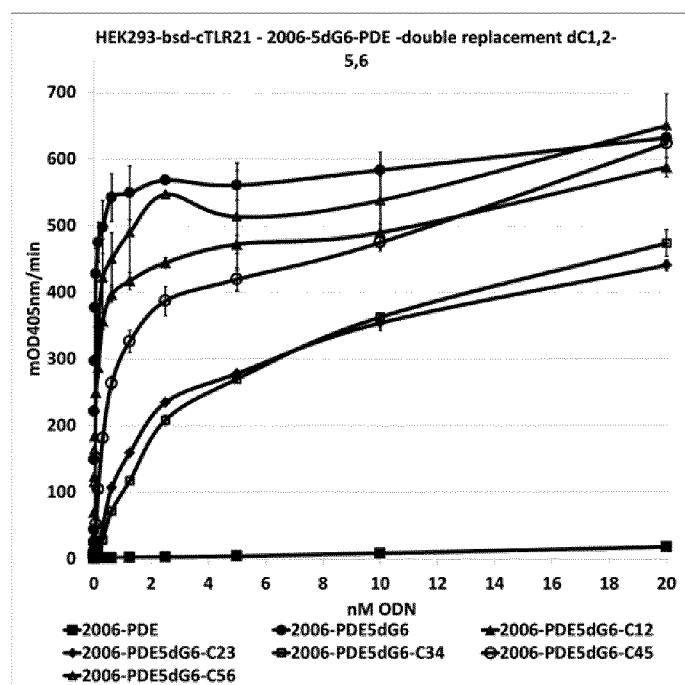


FIG. 12C

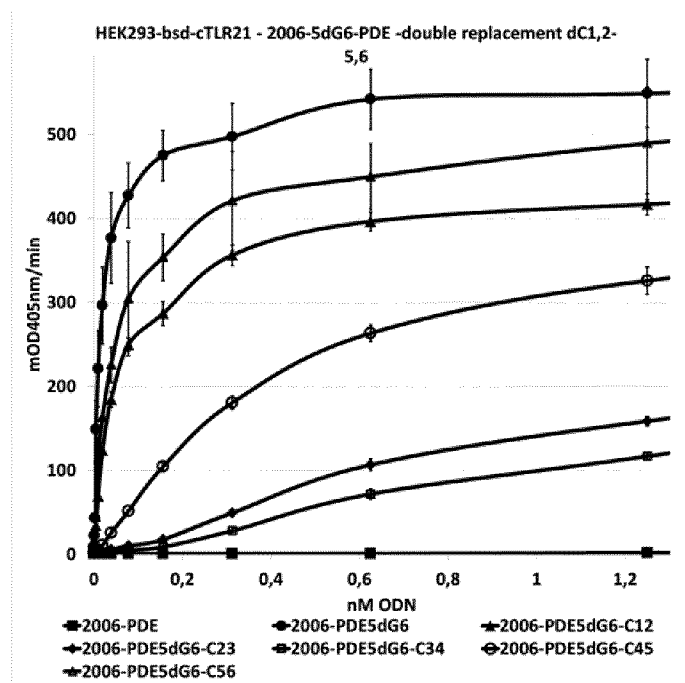


FIG. 12D

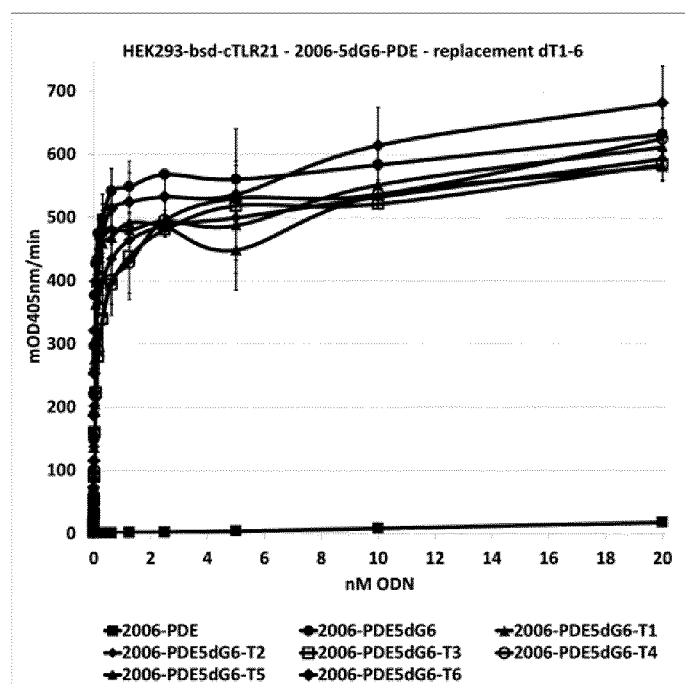


FIG. 13A

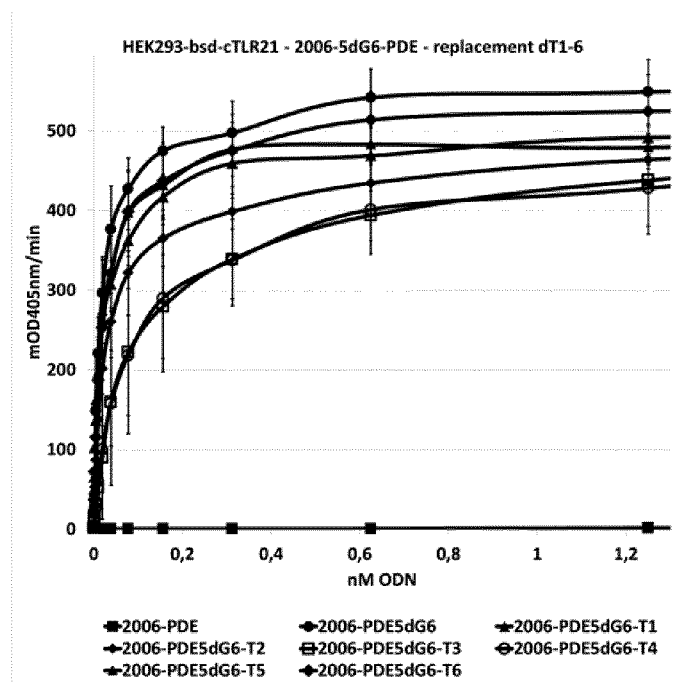


FIG. 13B

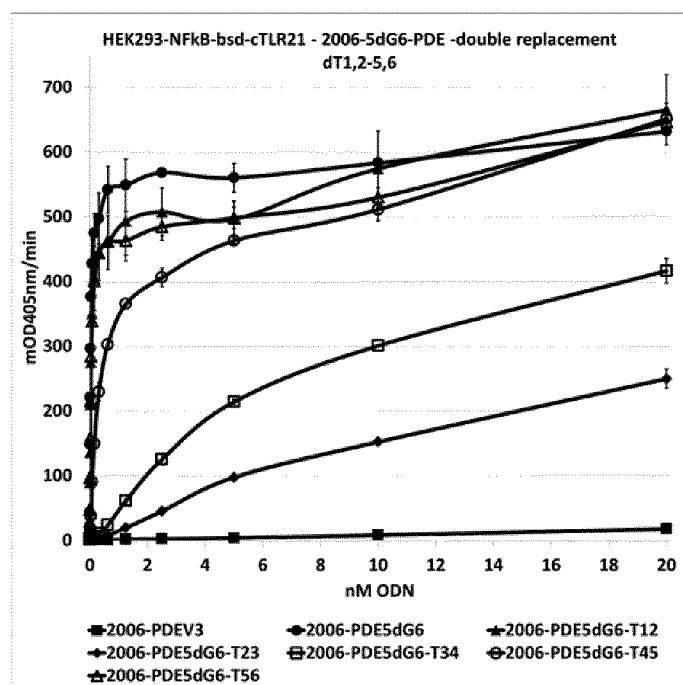


FIG. 13C

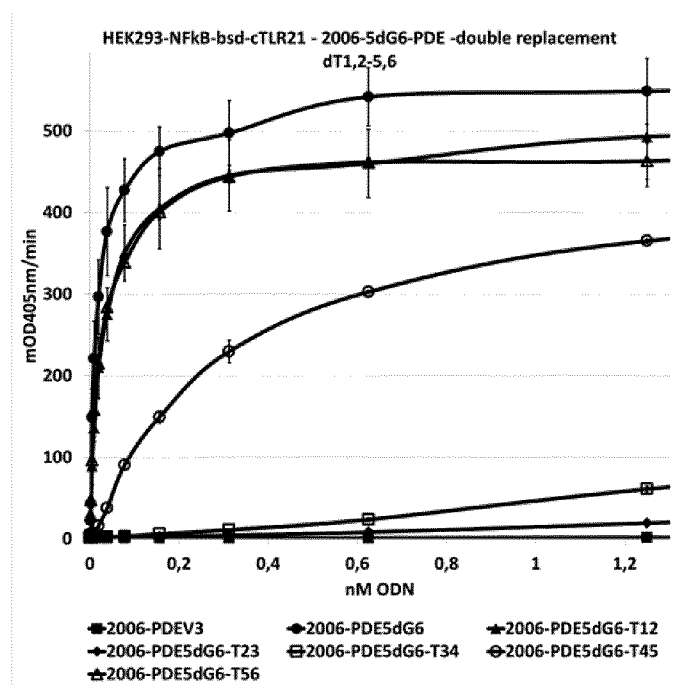
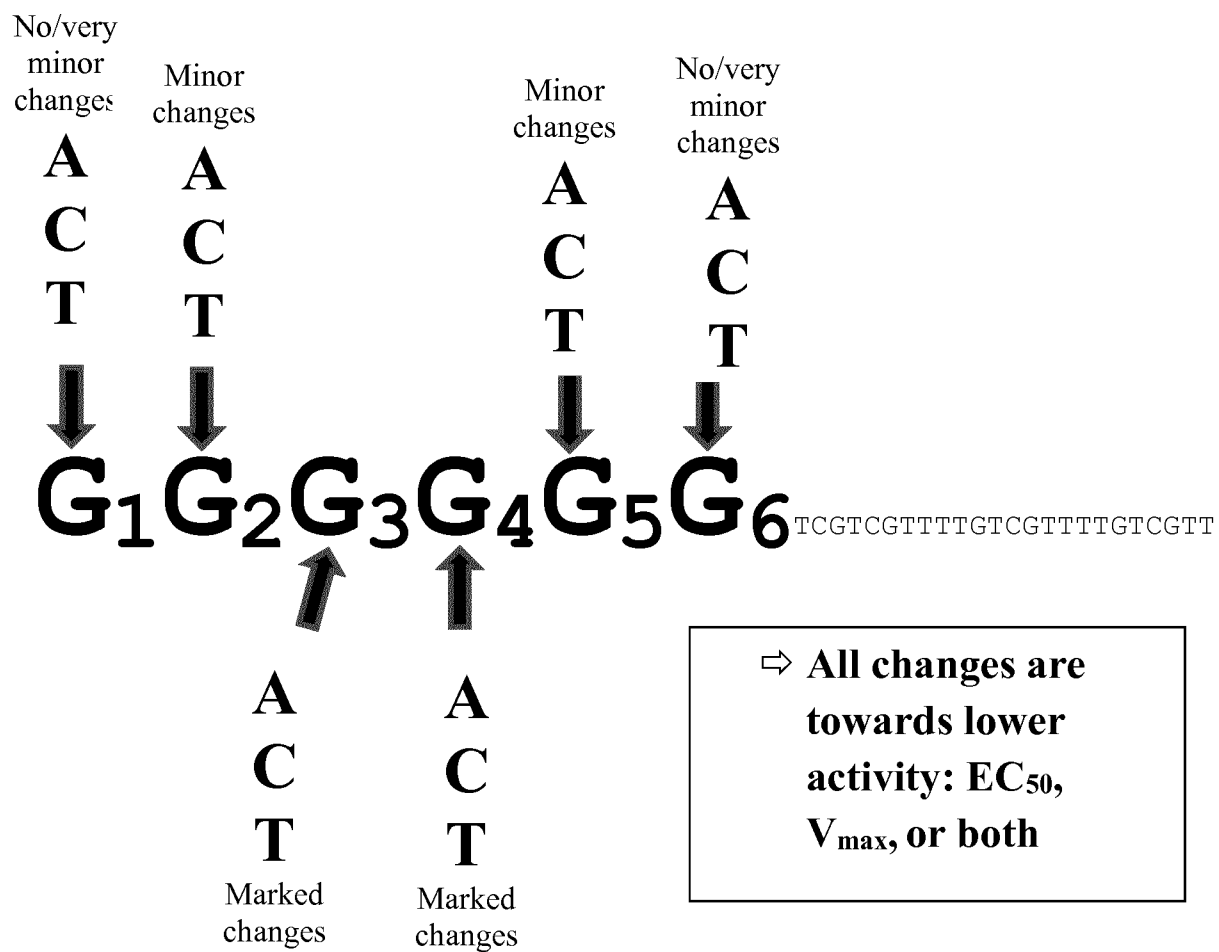
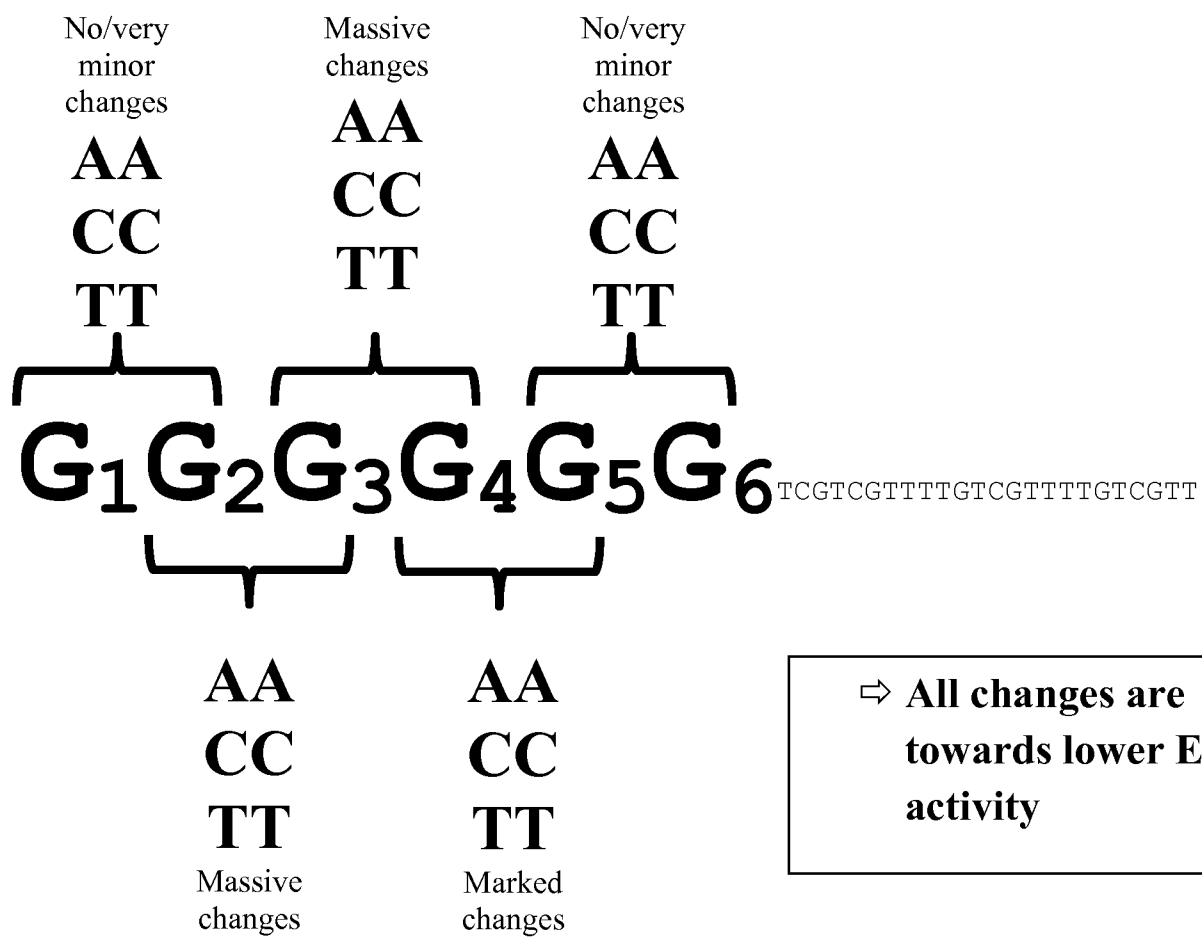


FIG. 13D

FIG. 14

Scheme 1:



Scheme 2:**FIG. 15**

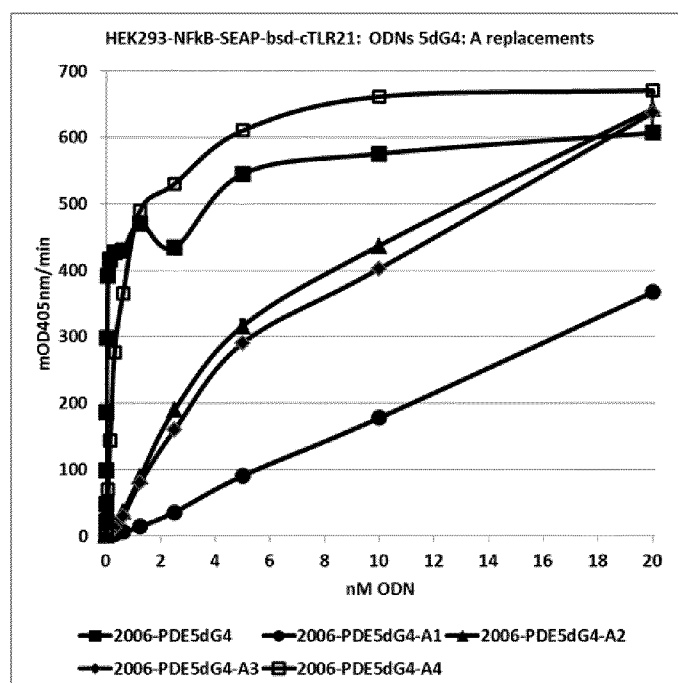


FIG. 16A

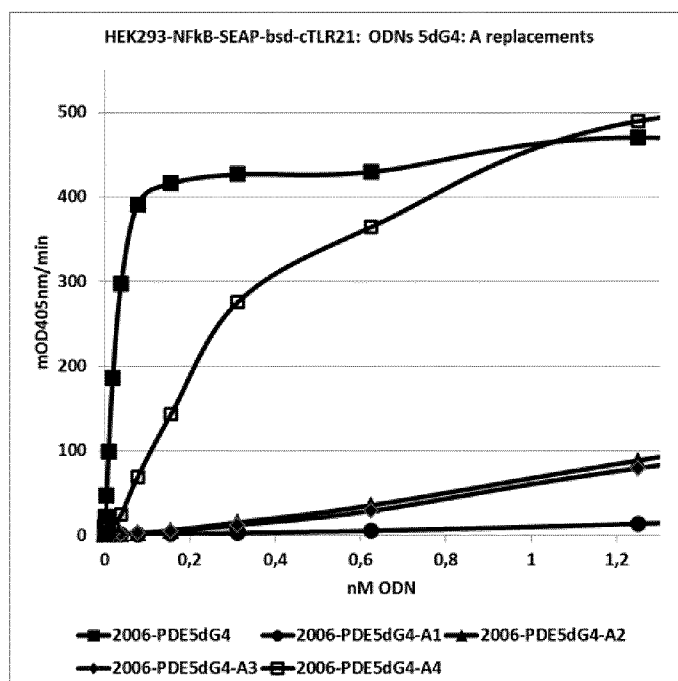


FIG. 16B

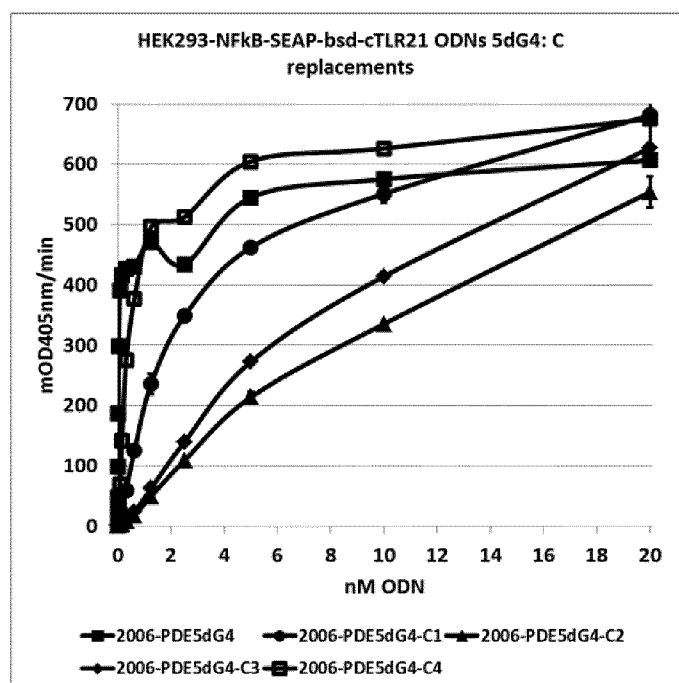


FIG. 17A

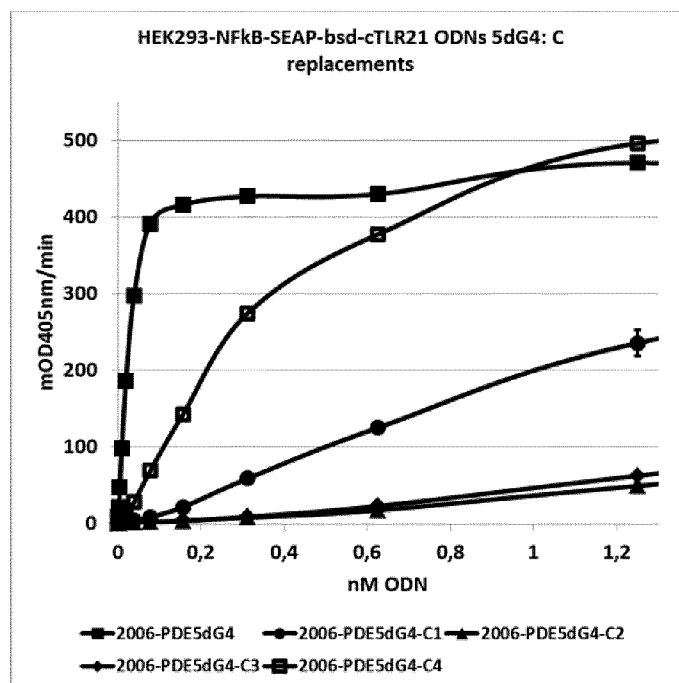


FIG. 17B

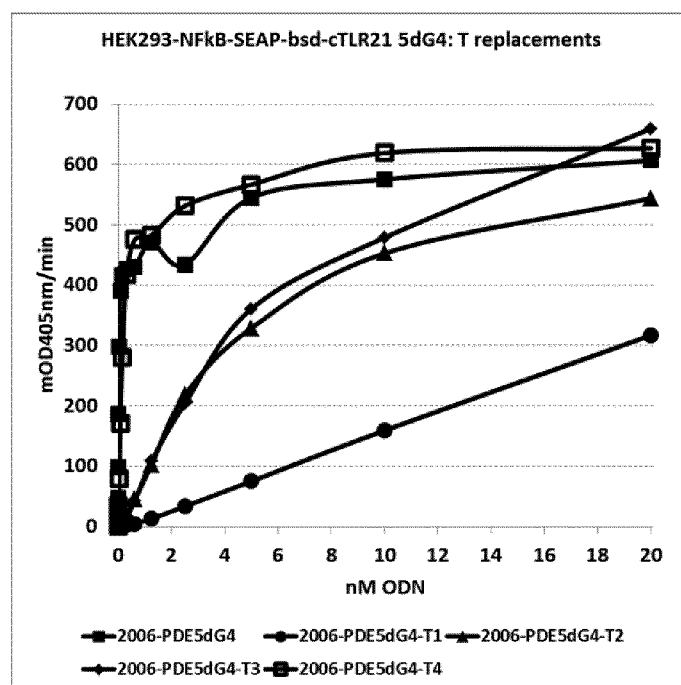


FIG. 18A

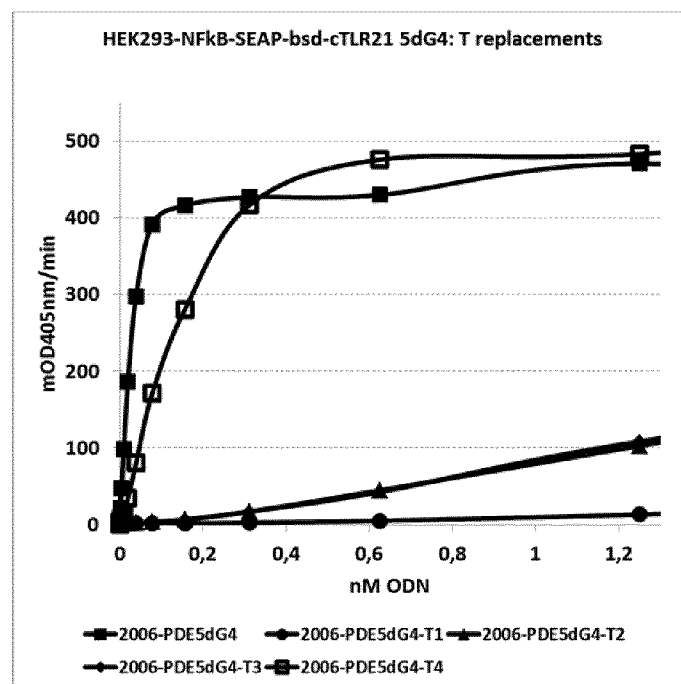


FIG. 18B

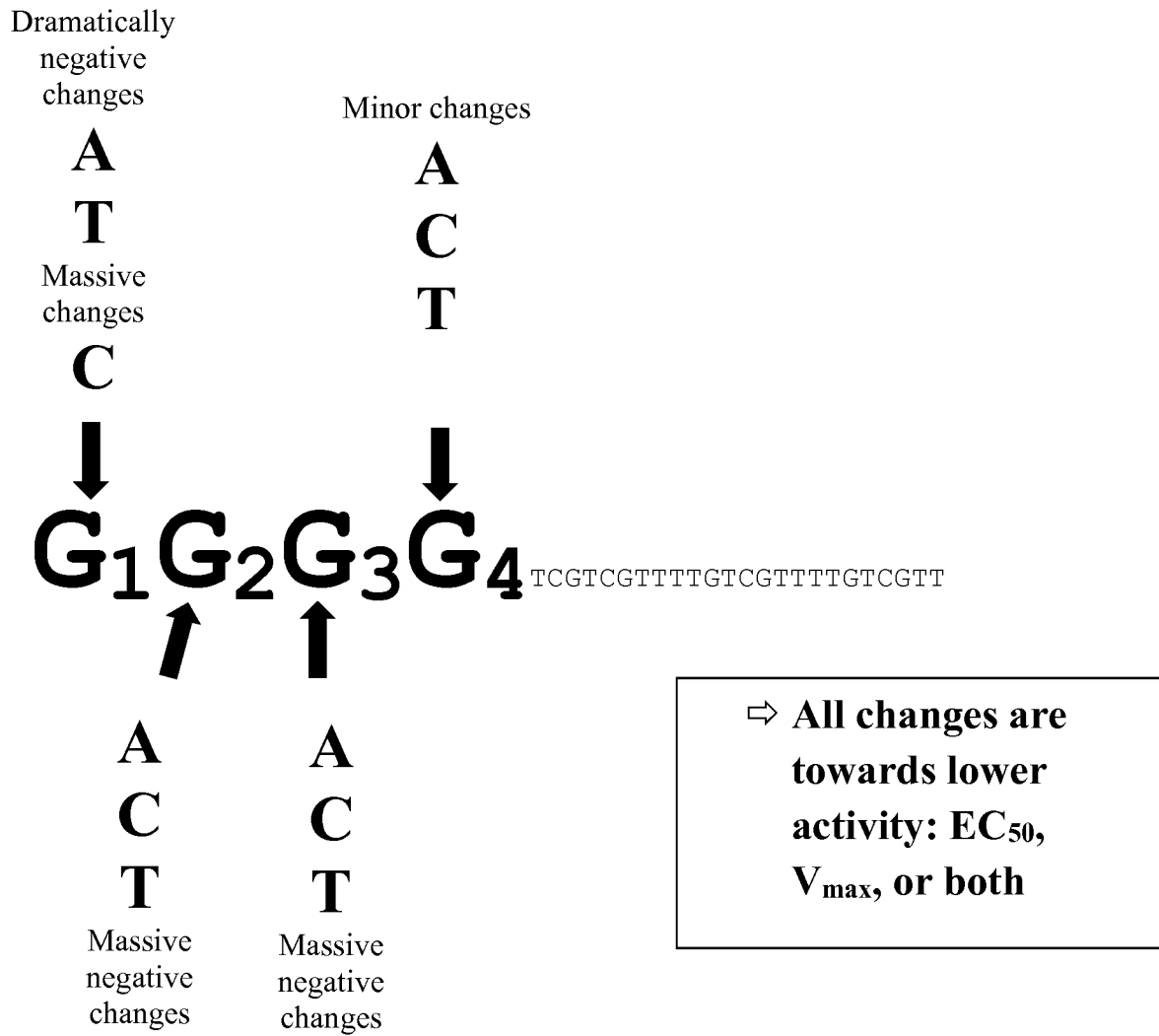


FIG. 19

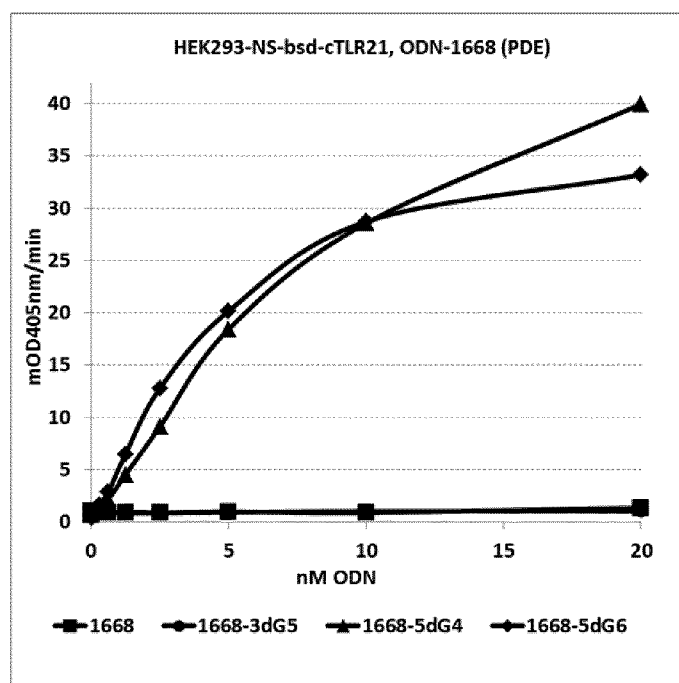


FIG. 20A

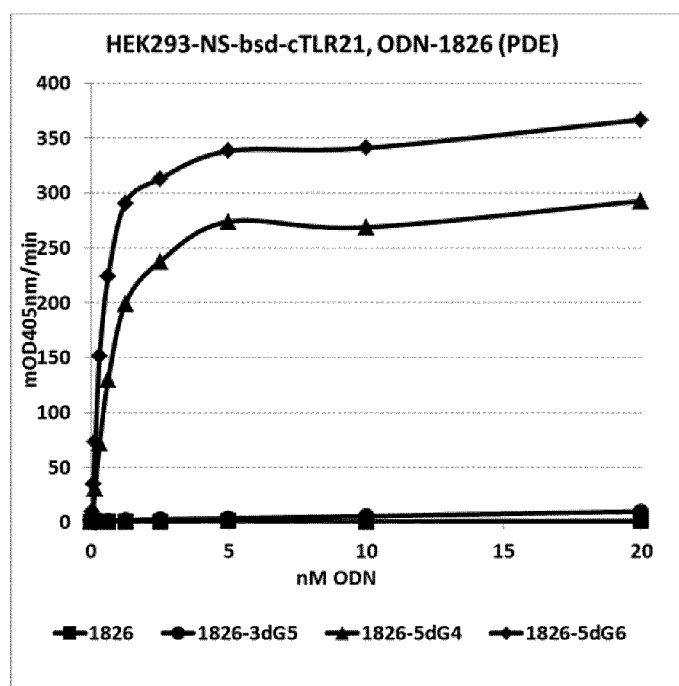


FIG. 20B

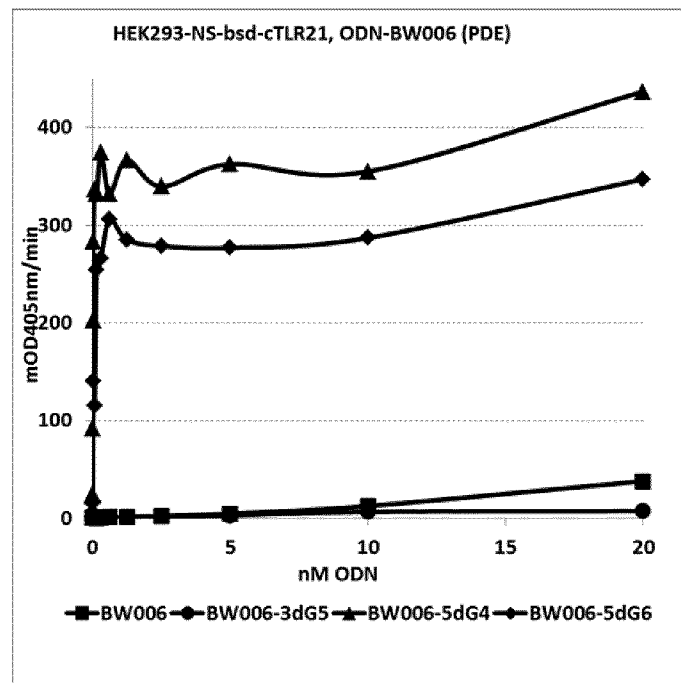


FIG. 20C

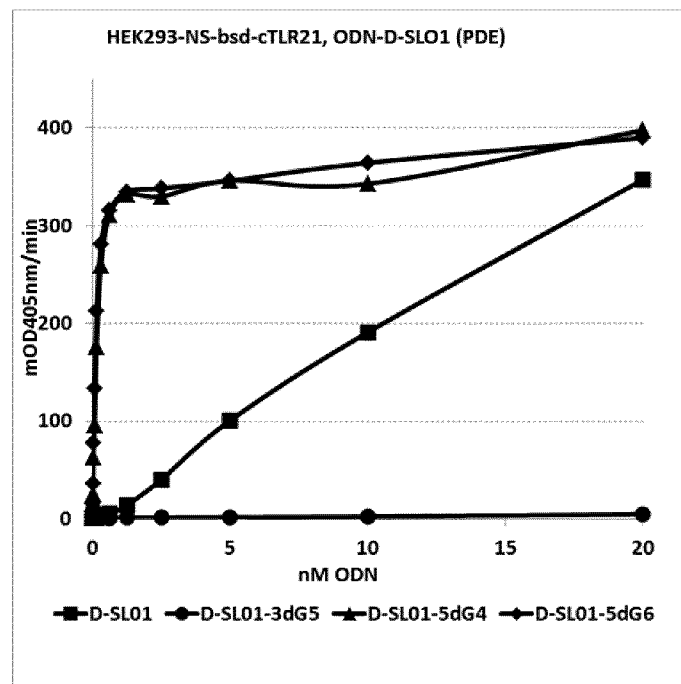


FIG. 20D

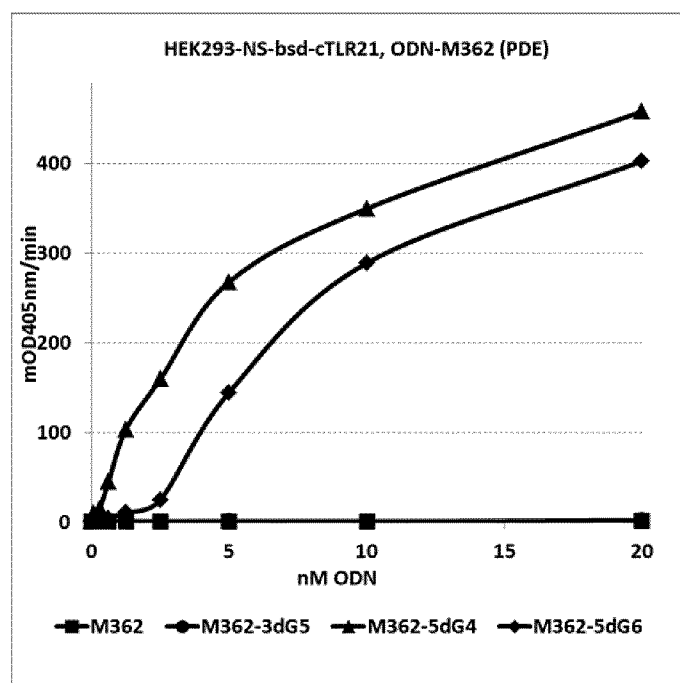


FIG. 20E

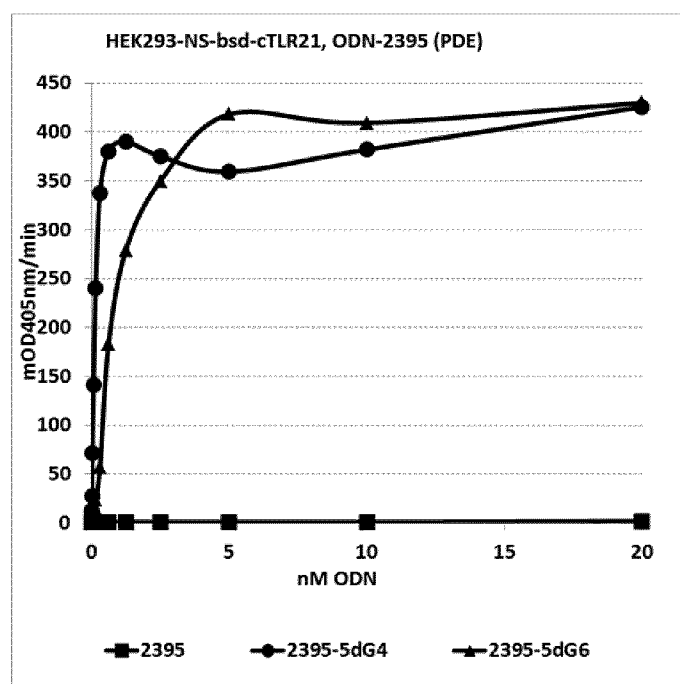


FIG. 20F

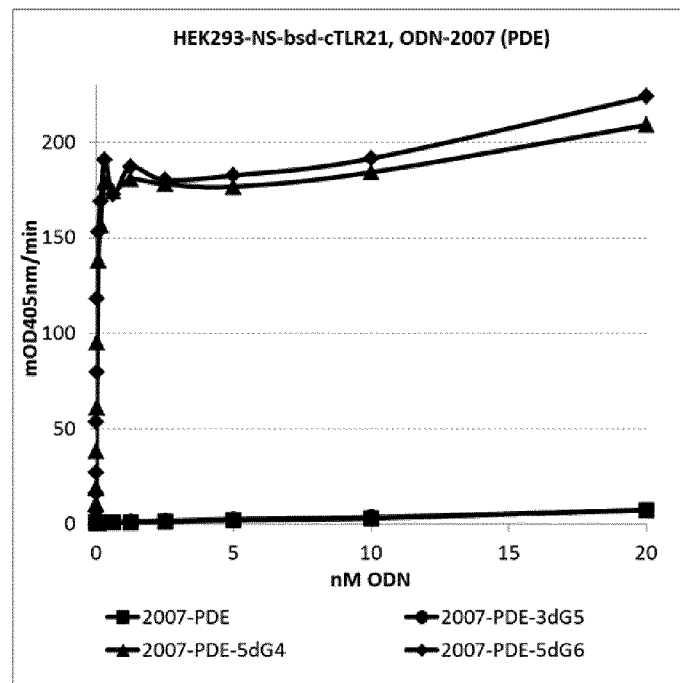


FIG. 20G

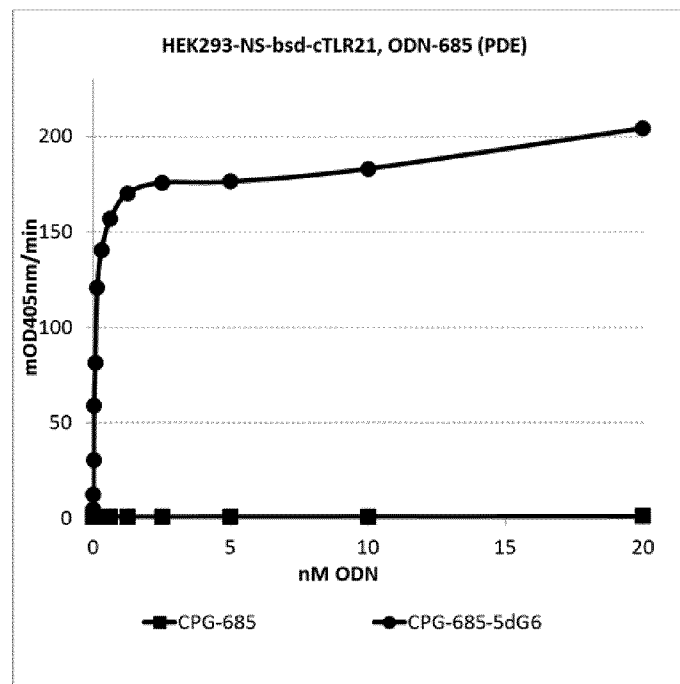


FIG. 20H

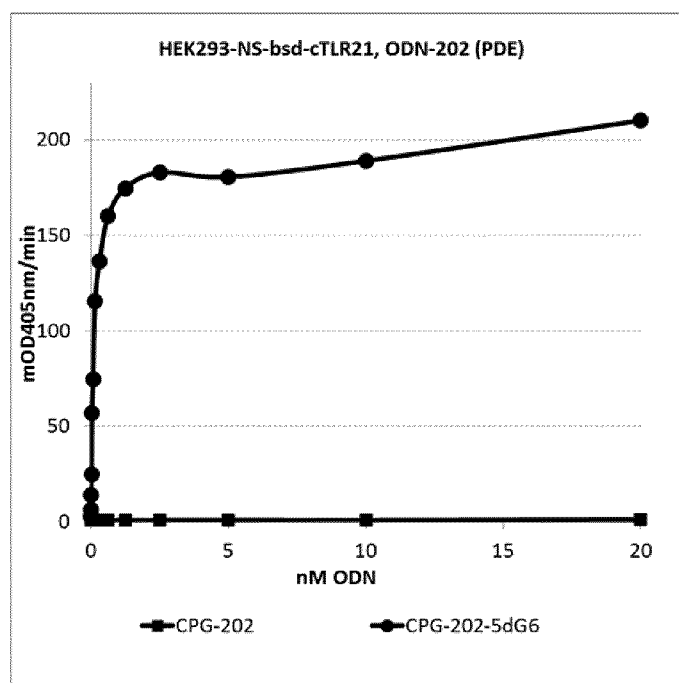


FIG. 20I

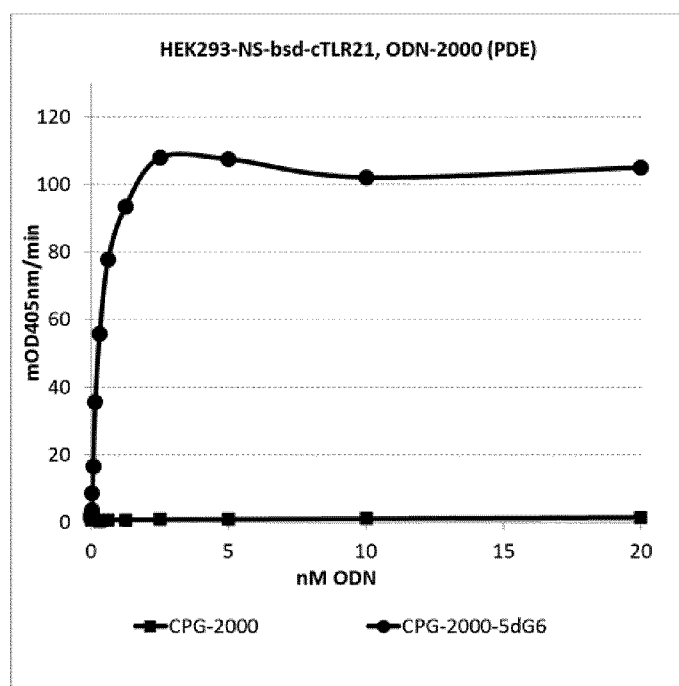
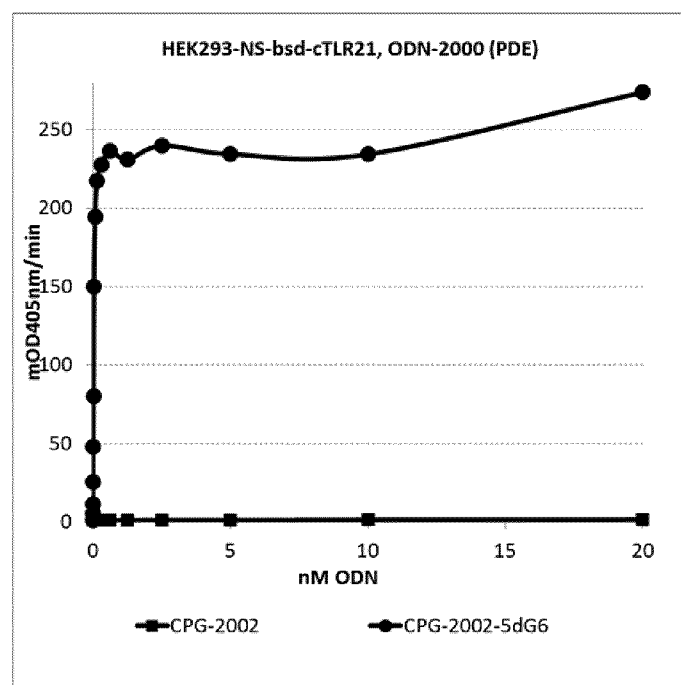


FIG. 20J

**FIG. 20K**

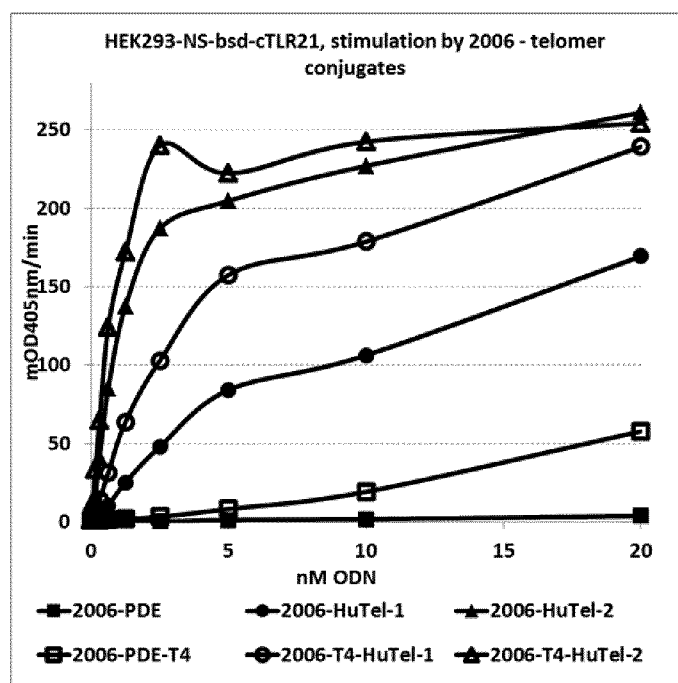


FIG. 21A

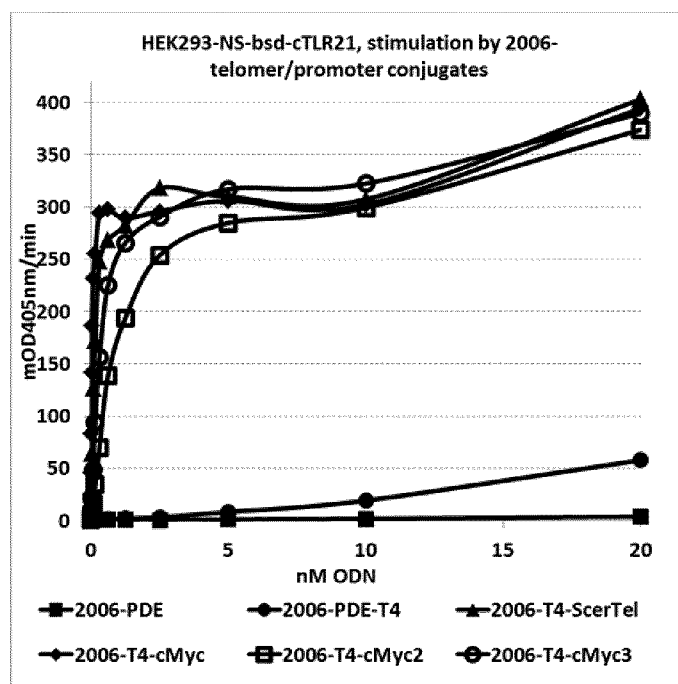


FIG. 21B

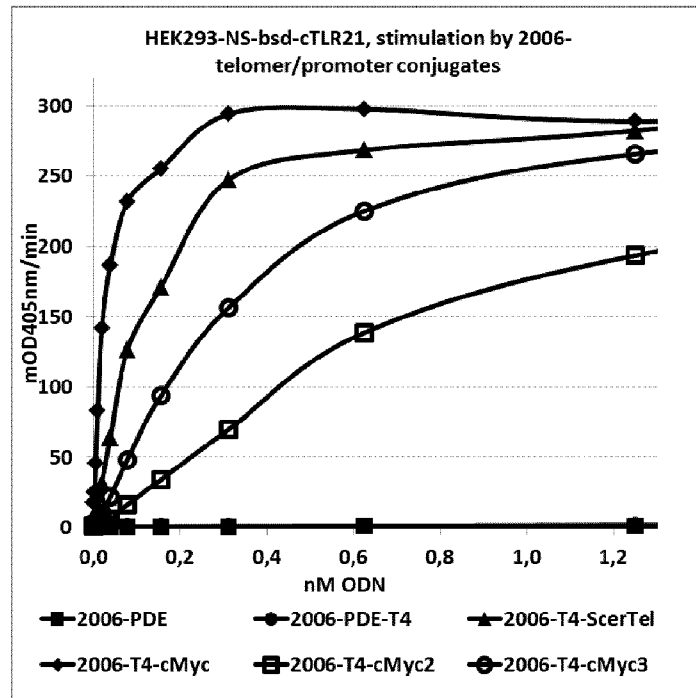


FIG. 21C

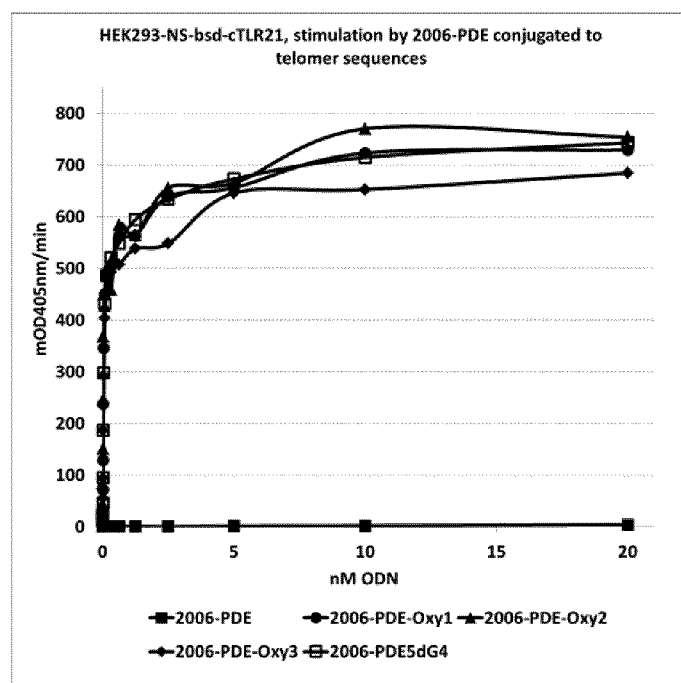


FIG. 22A

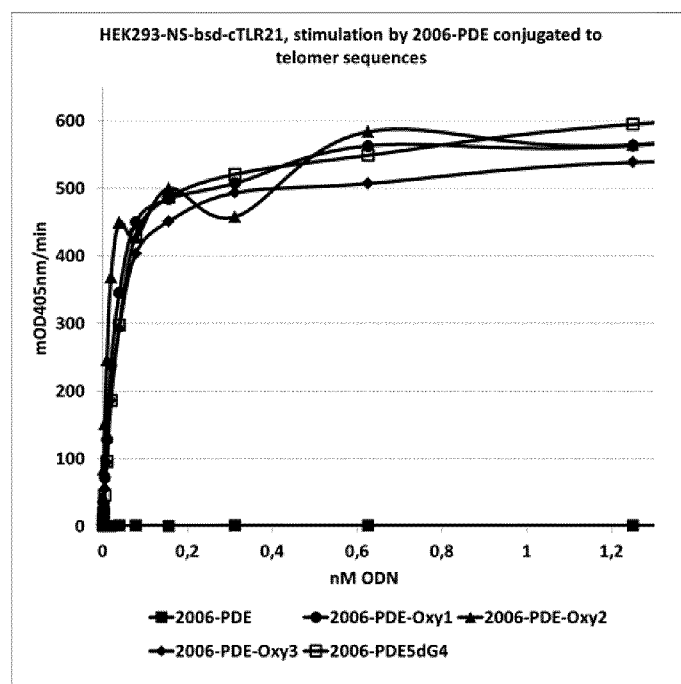


FIG. 22B

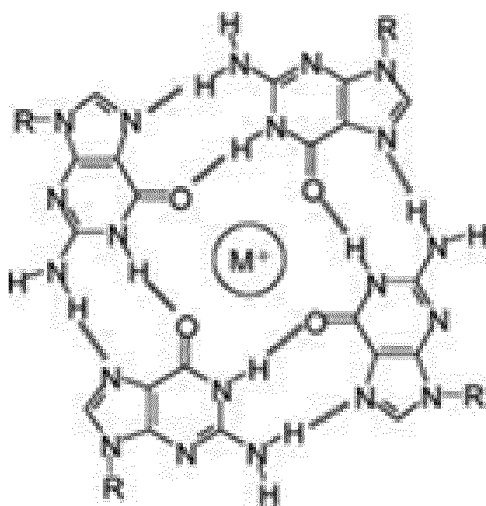


FIG. 23A

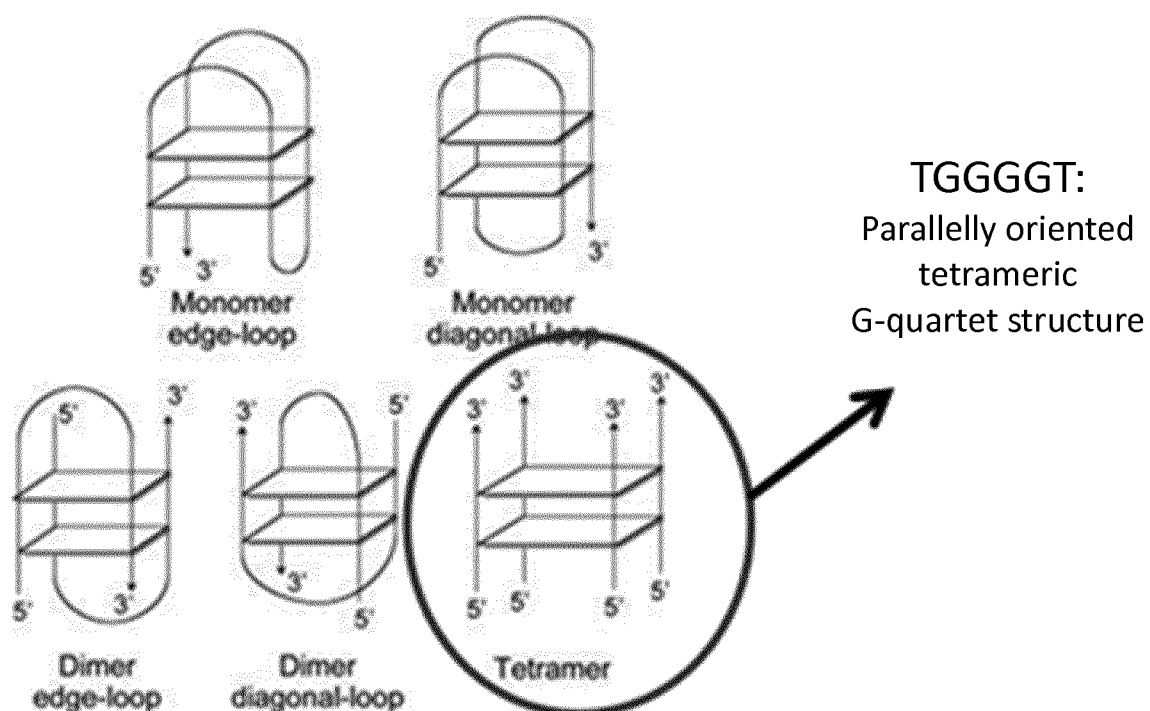


FIG. 23B

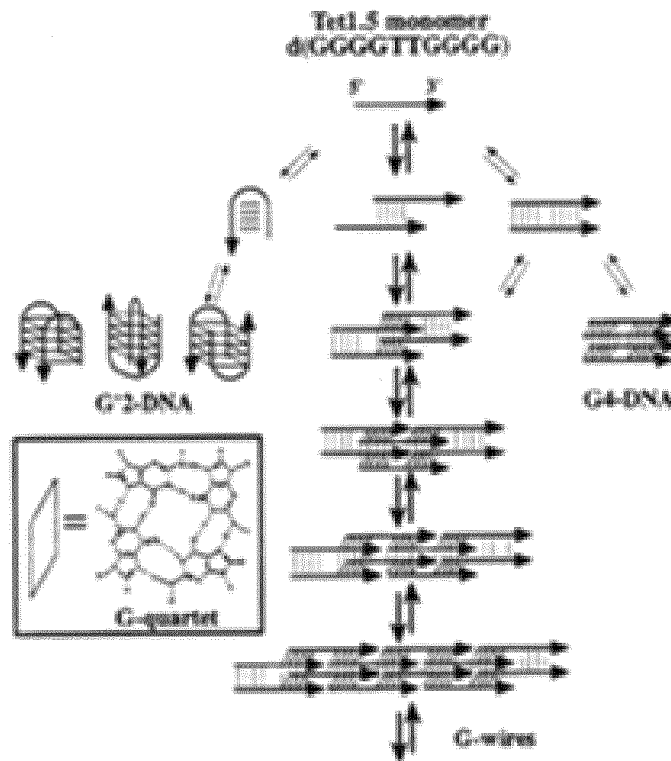
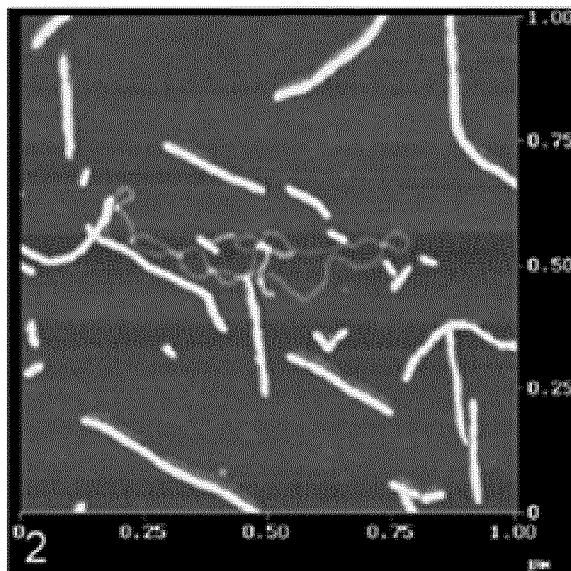


FIG. 23C



GGGGTTGGGG:
G-wire with the
potential to
"frayed" G-wire

FIG. 23D

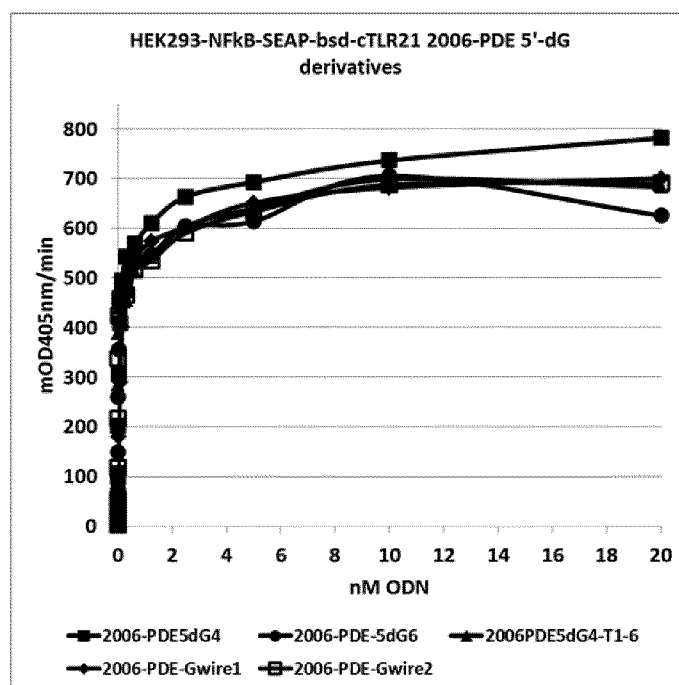


FIG. 24A

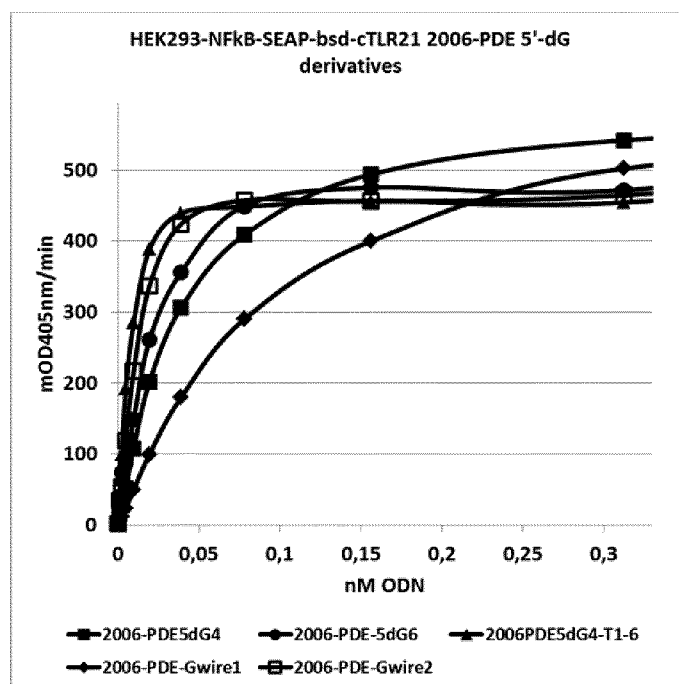


FIG. 24B

15% TBE-polyacrylamide gel 1:

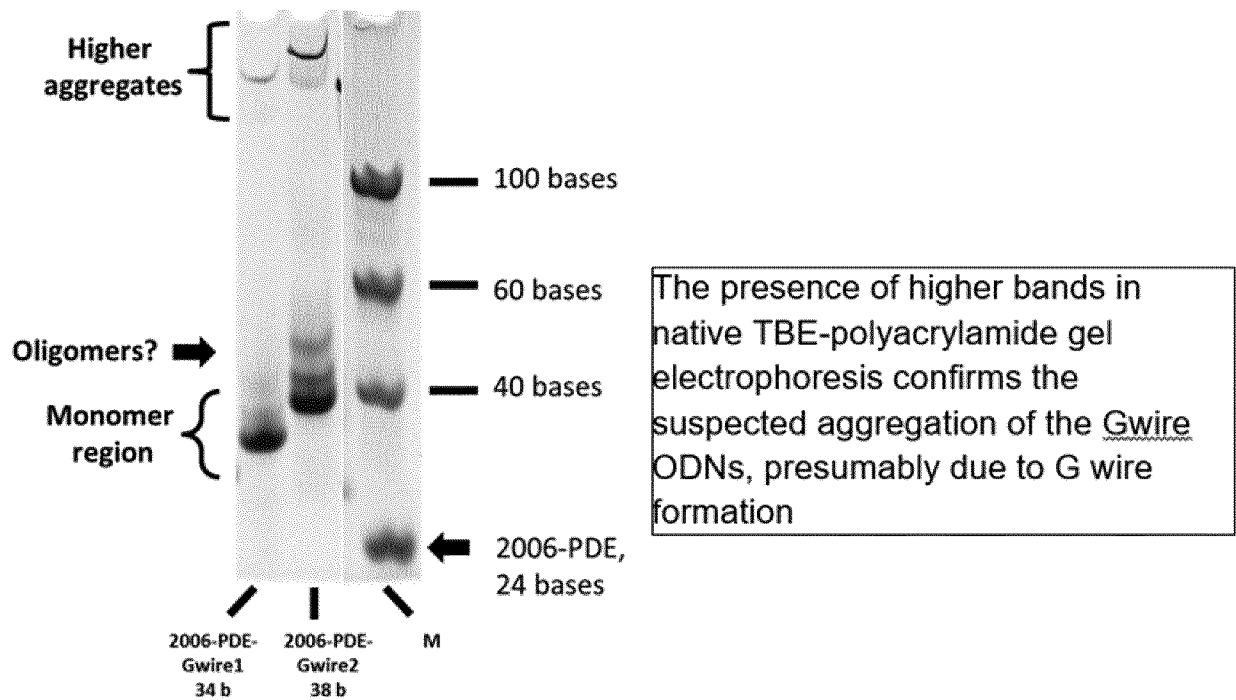


FIG. 25

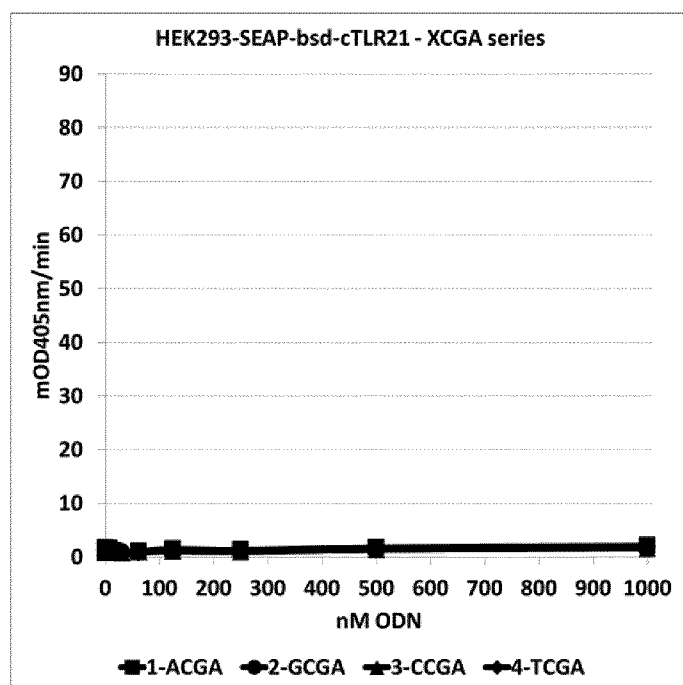


FIG. 26A

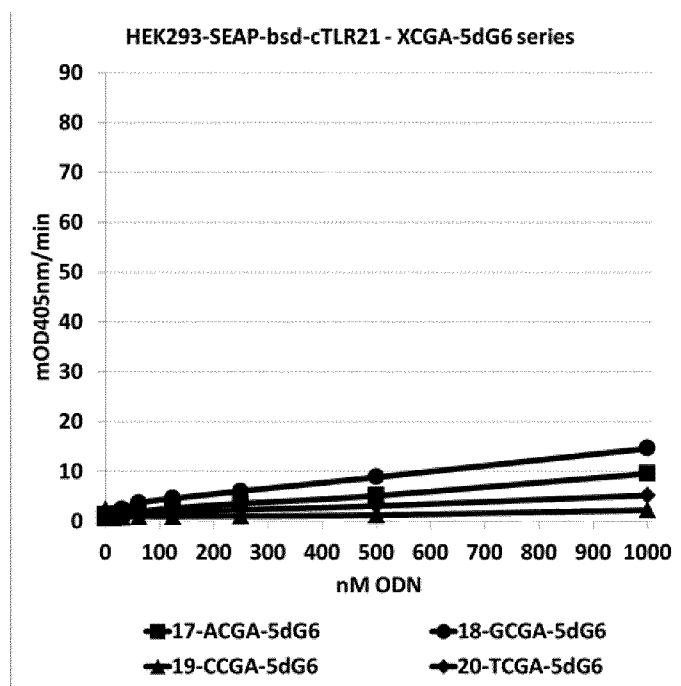


FIG. 26B

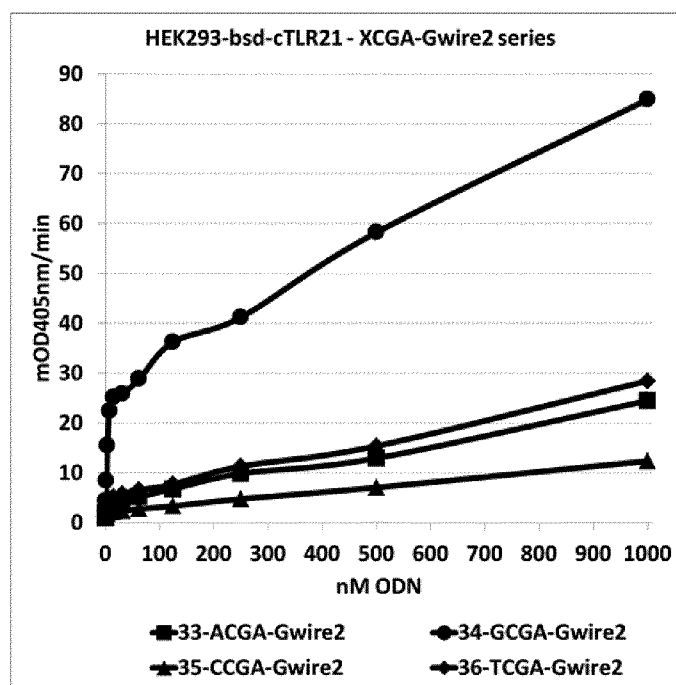


FIG. 26C

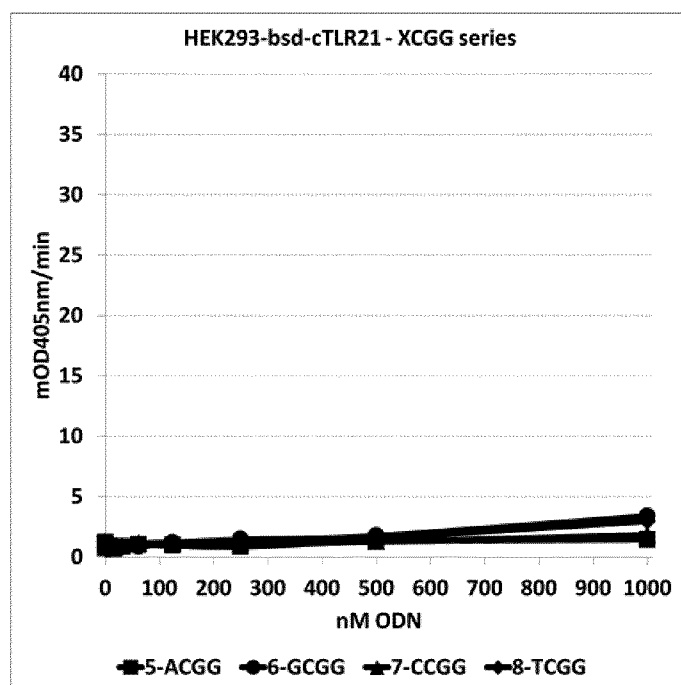


FIG. 27A

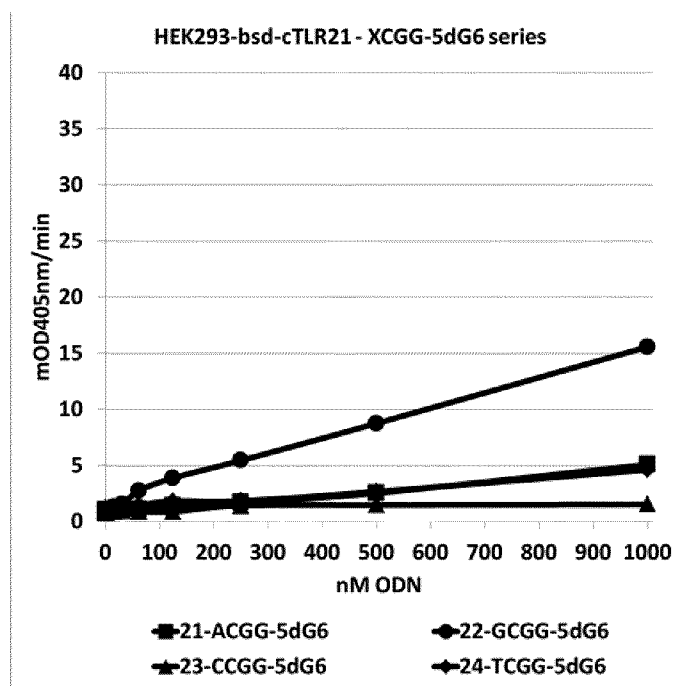


FIG. 27B

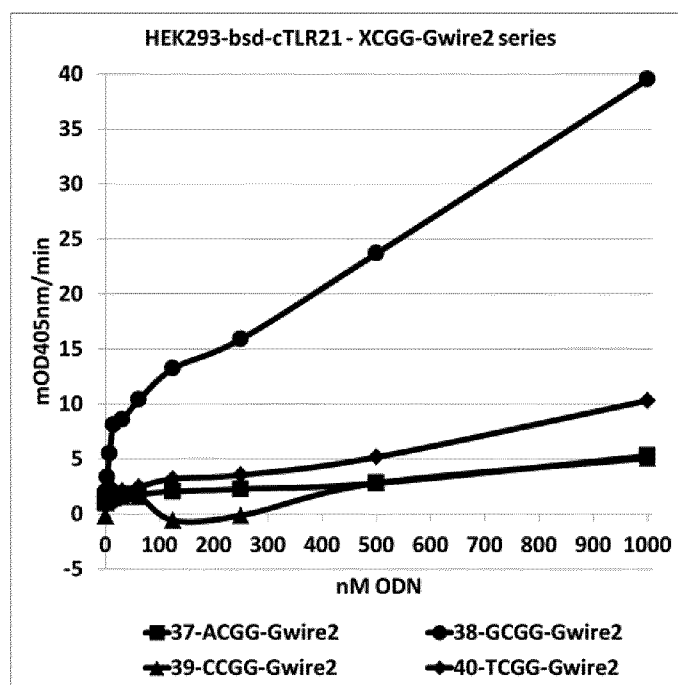


FIG. 27C

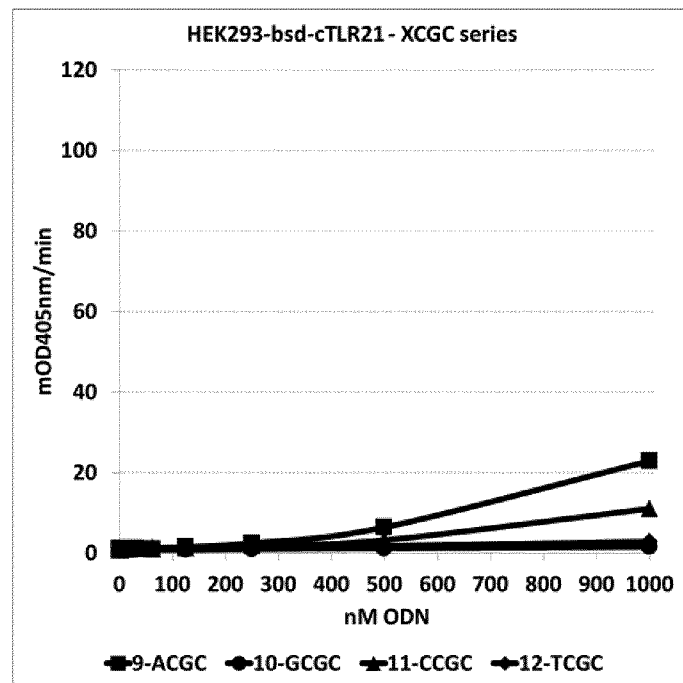


FIG. 28A

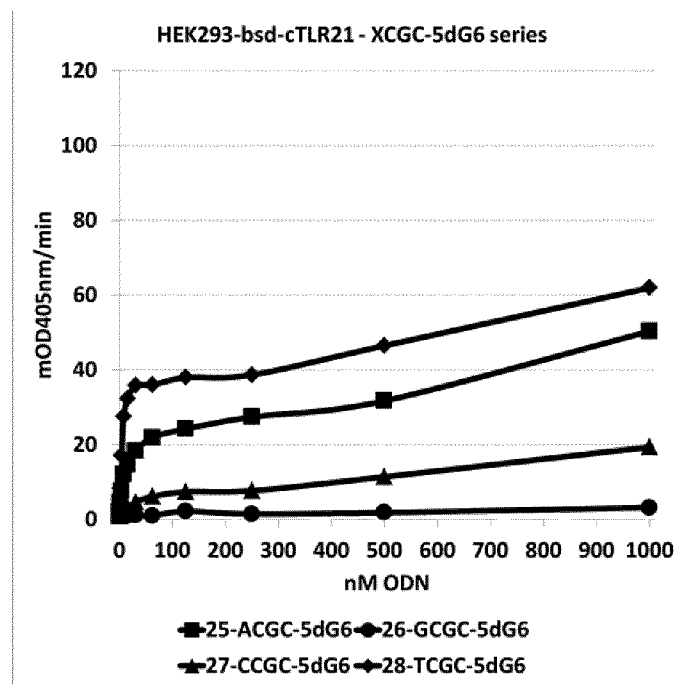


FIG. 28B

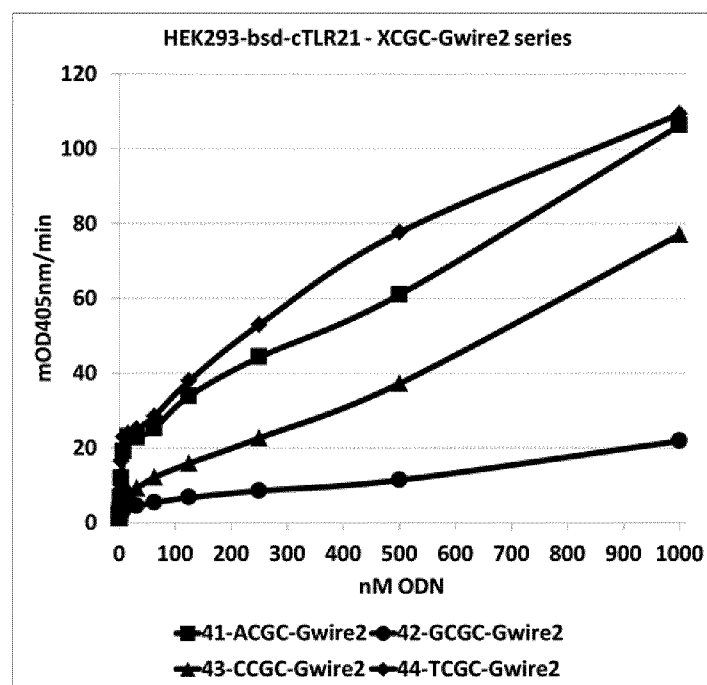


FIG. 28C

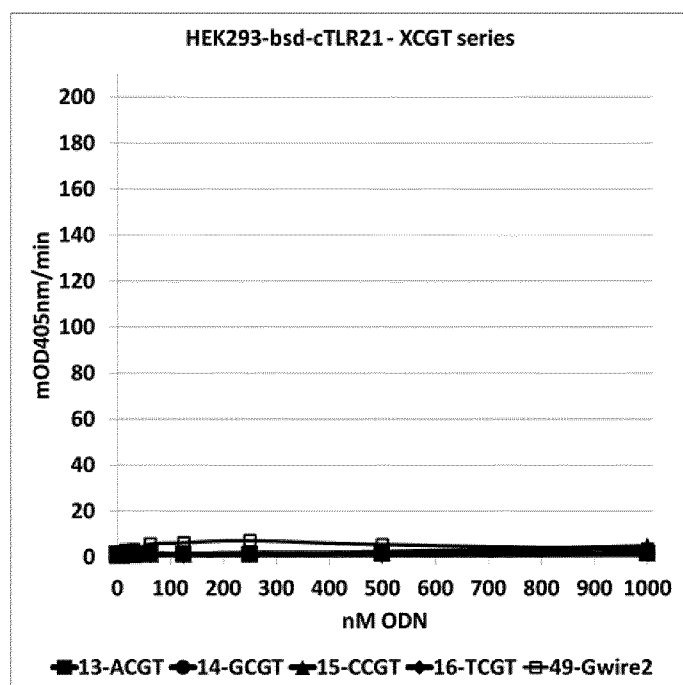


FIG. 29A

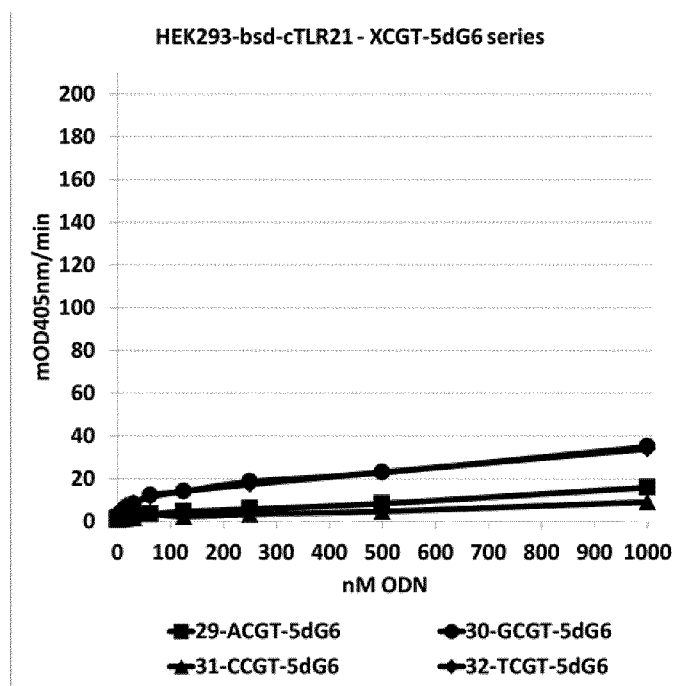


FIG. 29B

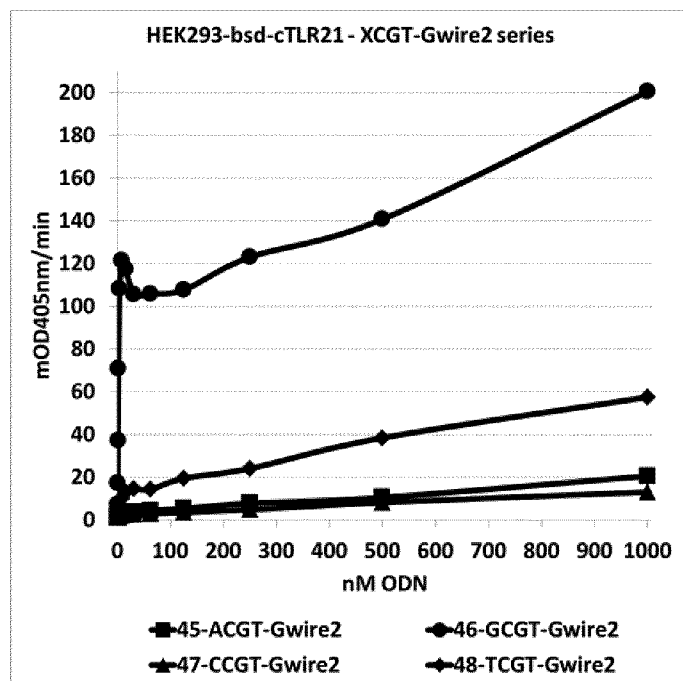


FIG. 29C

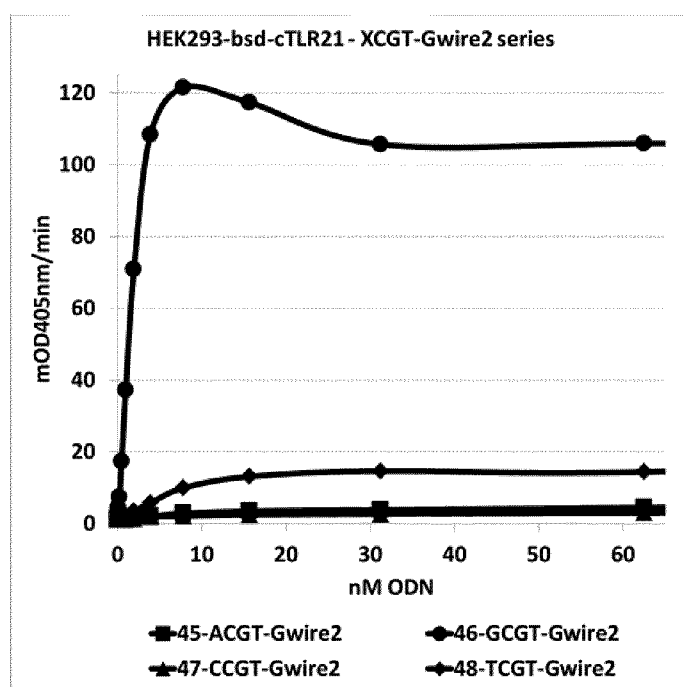


FIG. 29D

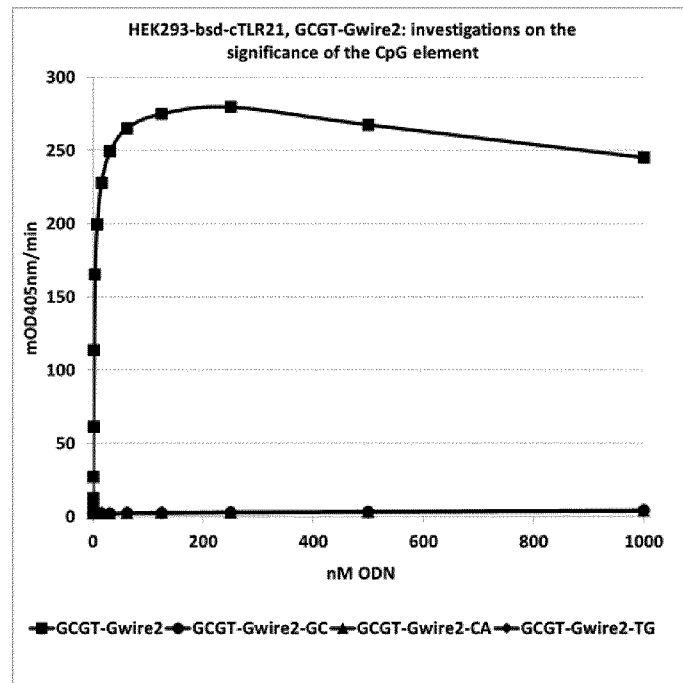


FIG. 30

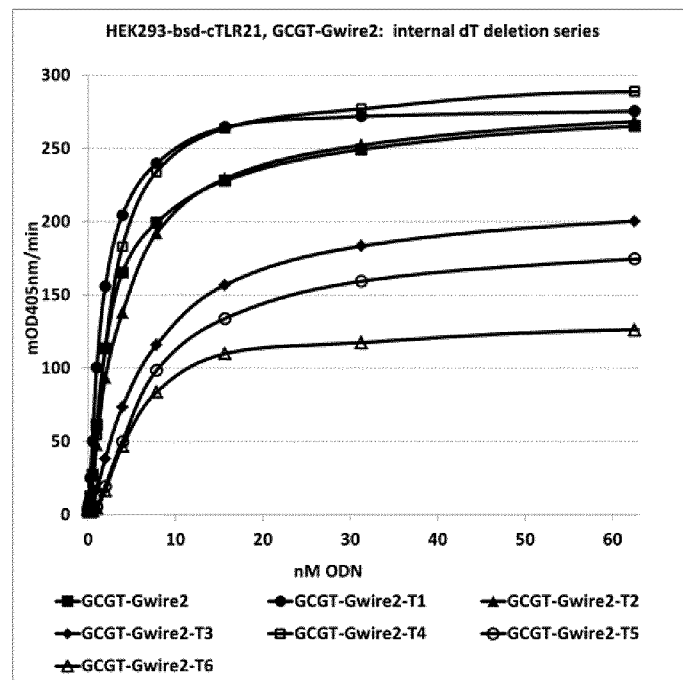


FIG. 31

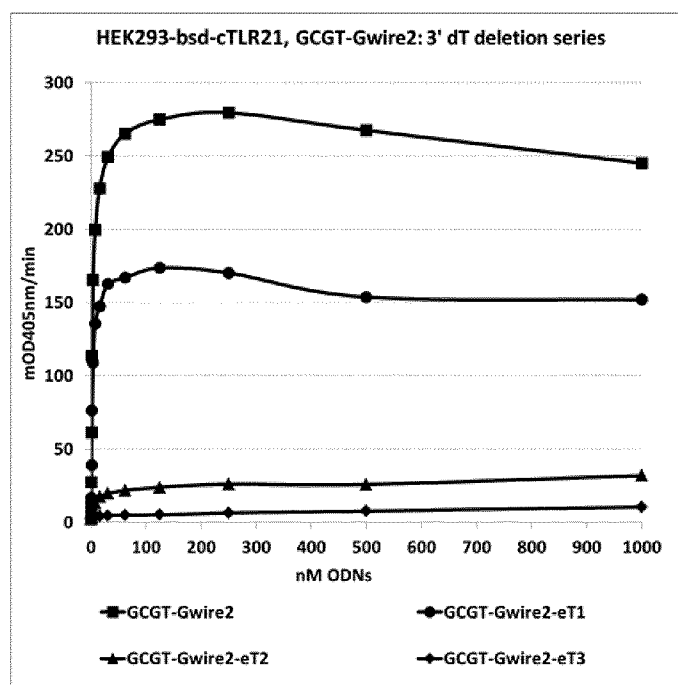


FIG. 32

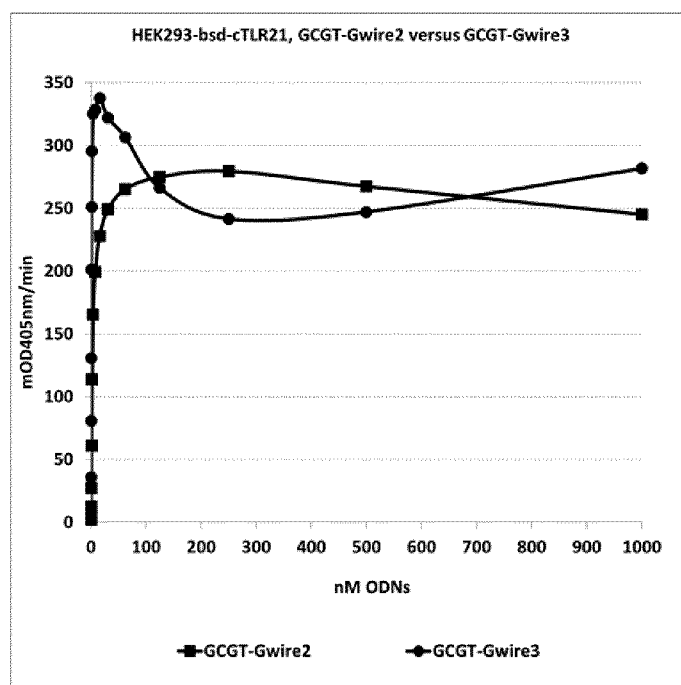


FIG. 33A

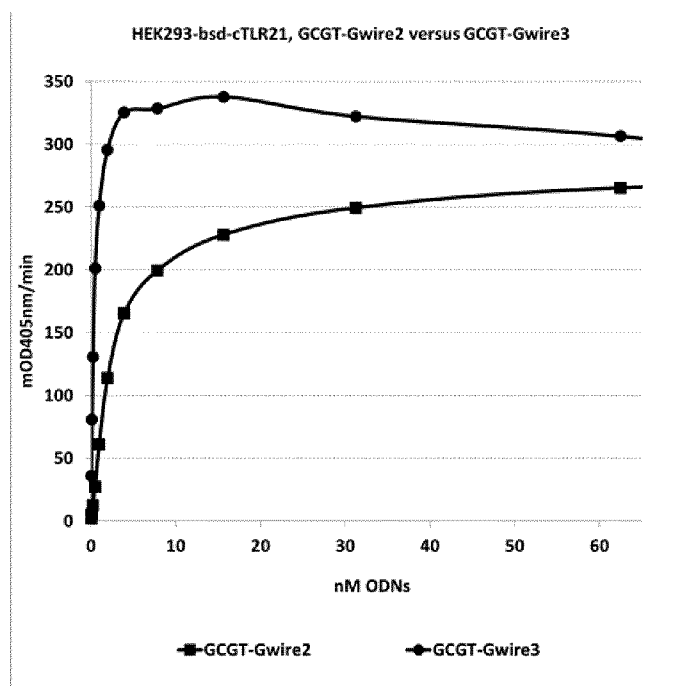


FIG. 33B

Scheme: Preliminary SAR of GCGT-Gwire2:

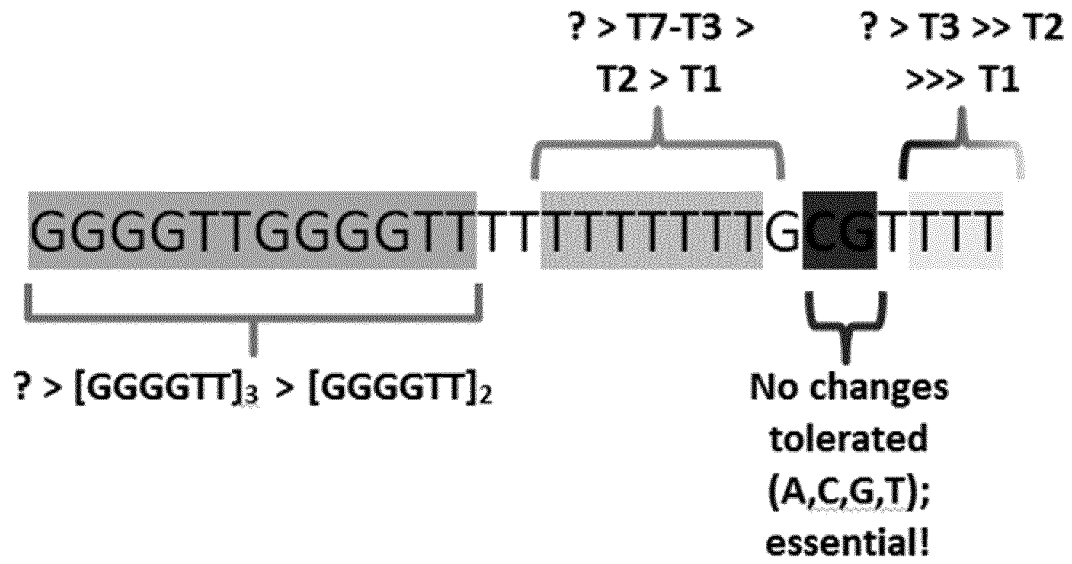


FIG. 34

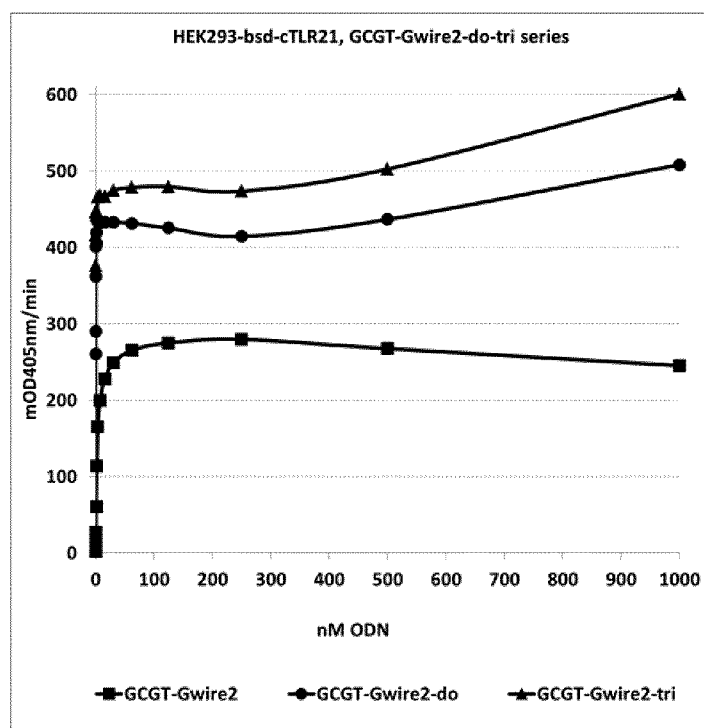


FIG. 35A

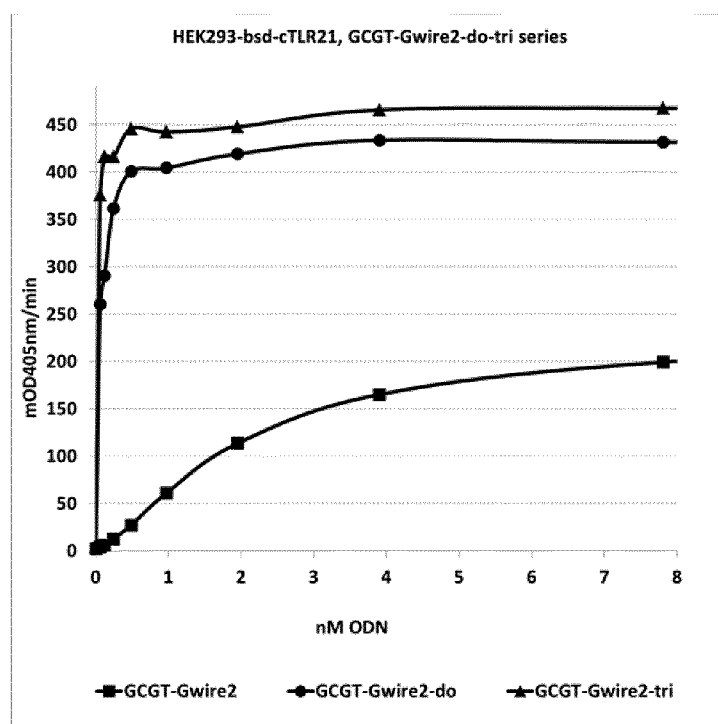


FIG. 35B

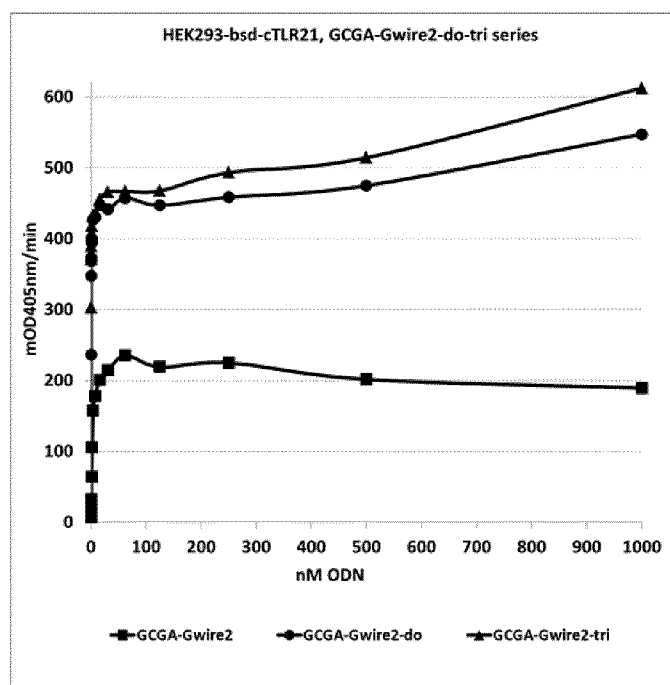


FIG. 36A

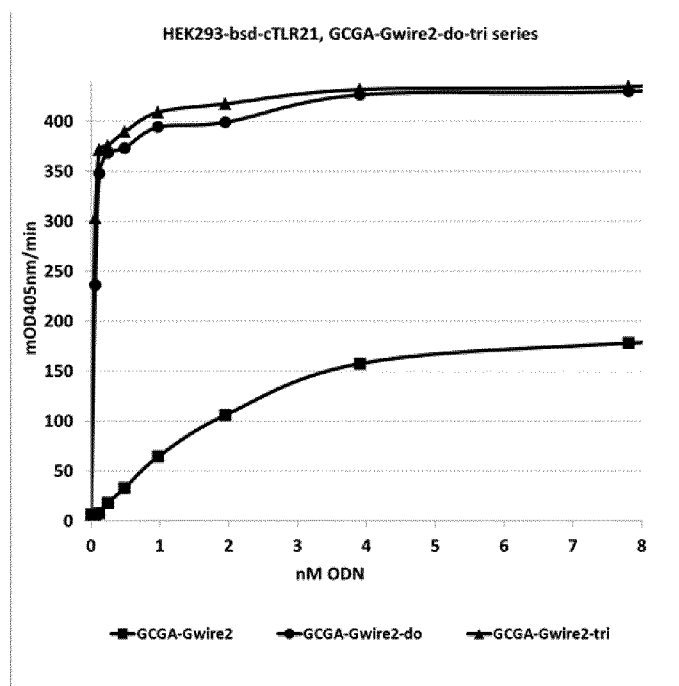


FIG. 36B

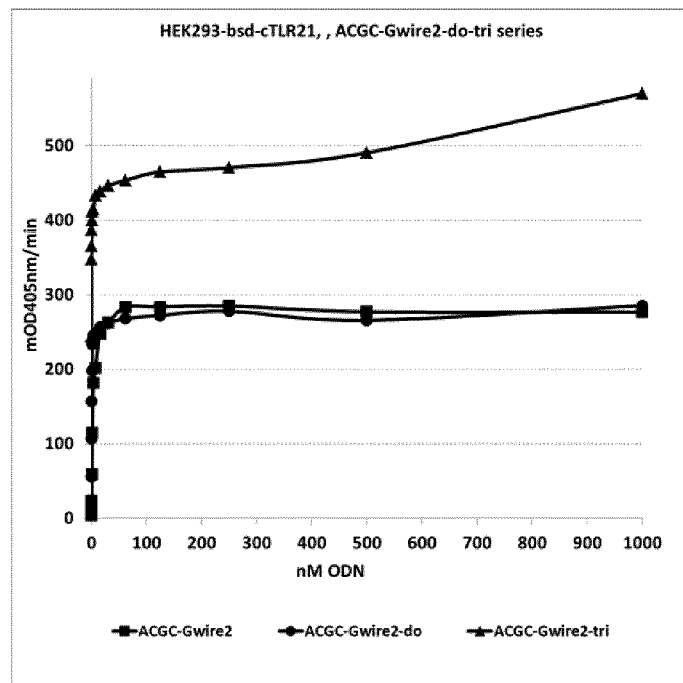


FIG. 37A

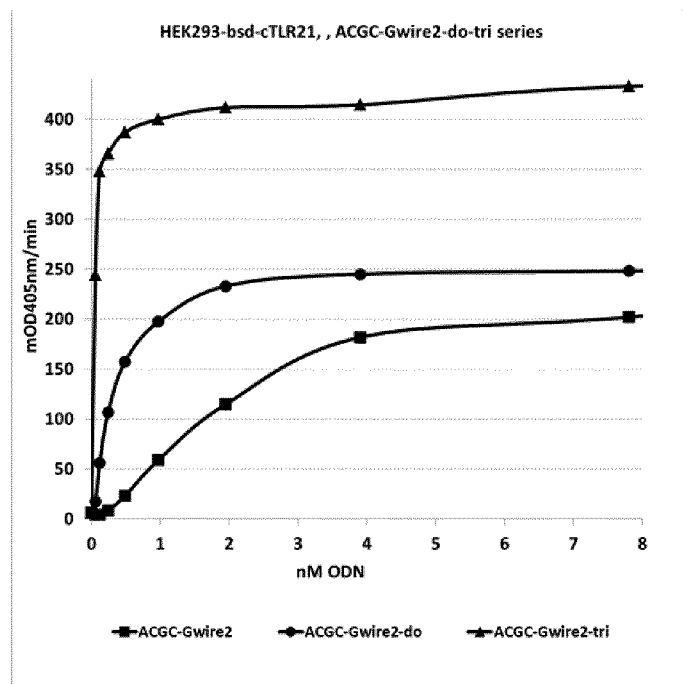


FIG. 37B

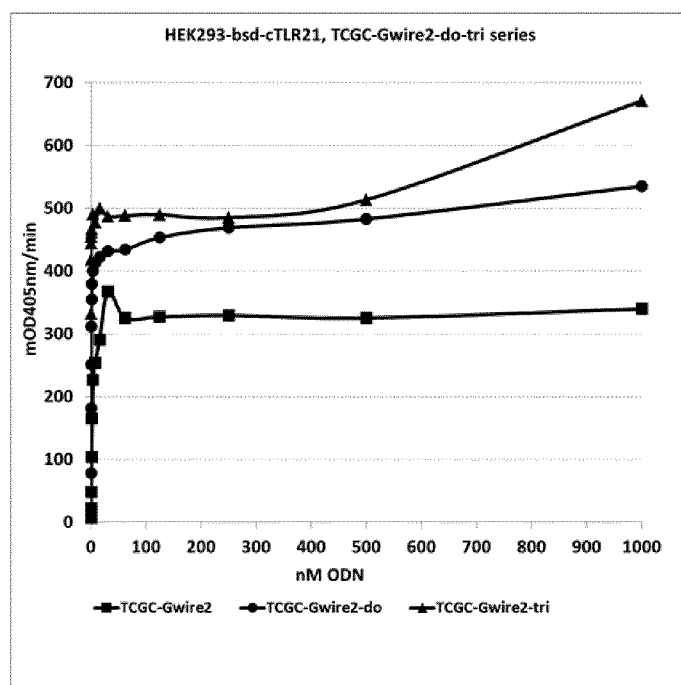


FIG. 38A

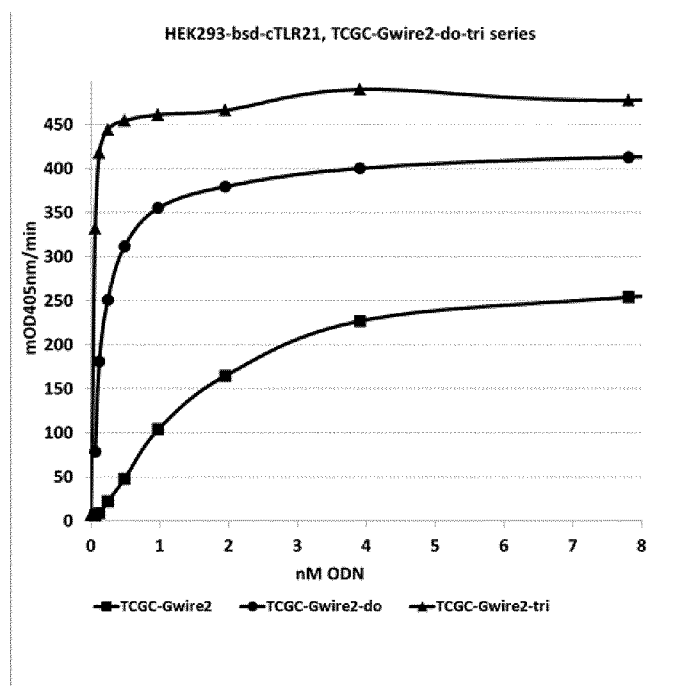


FIG. 38B

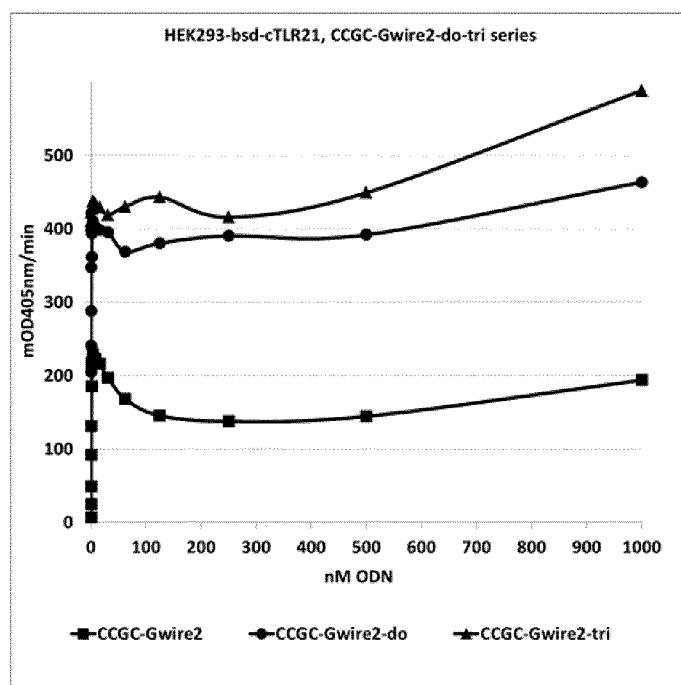


FIG. 39A

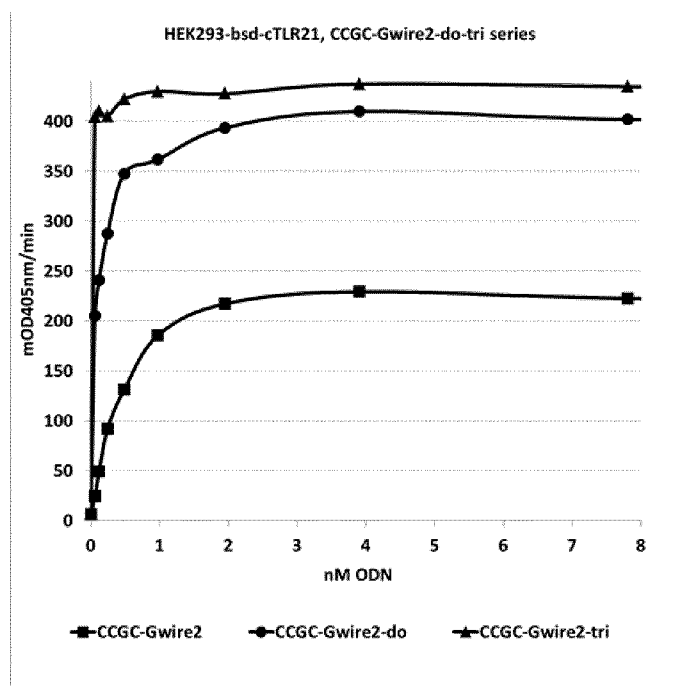


FIG. 39B

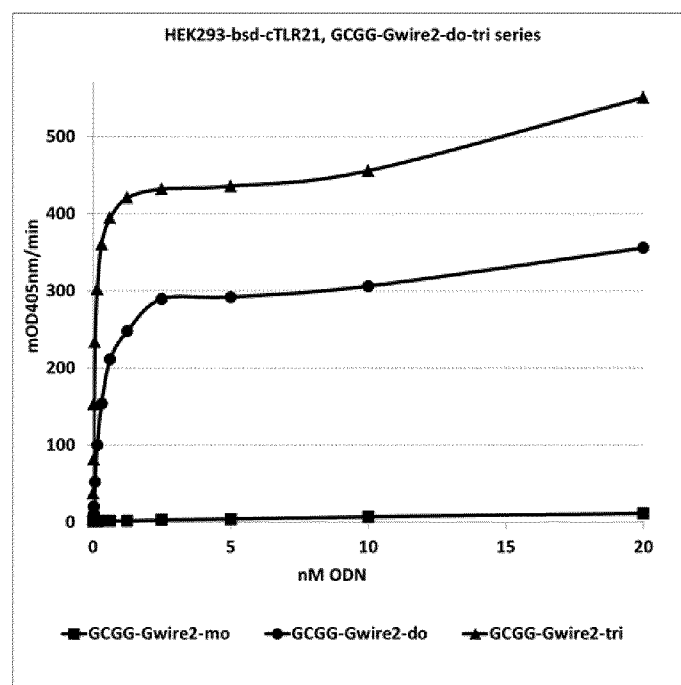


FIG. 40

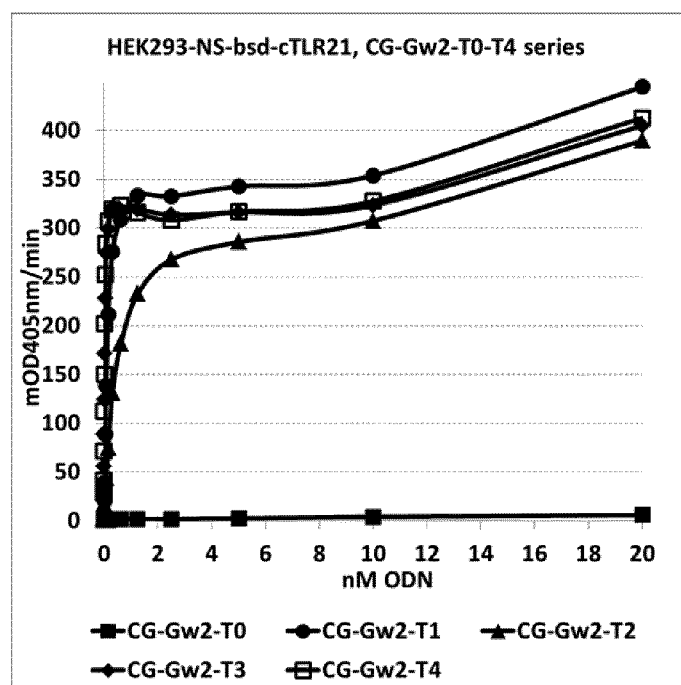


FIG. 41A

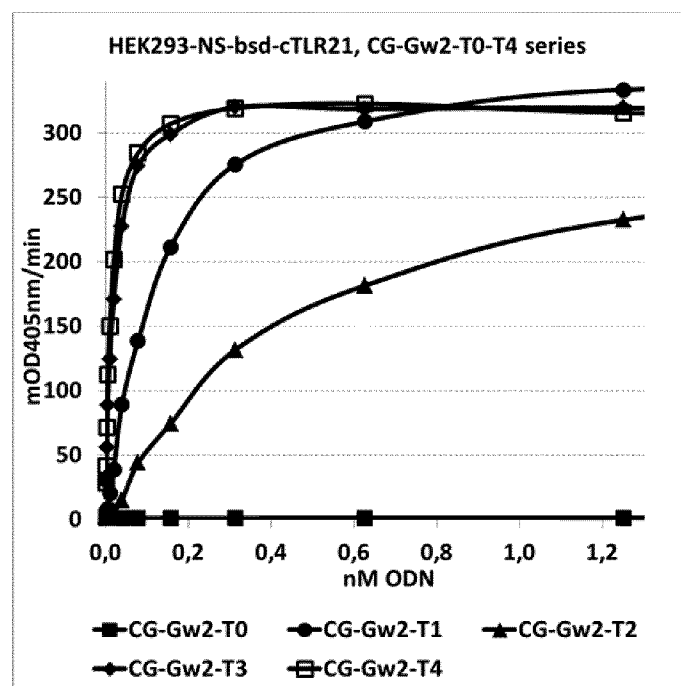


FIG. 41B

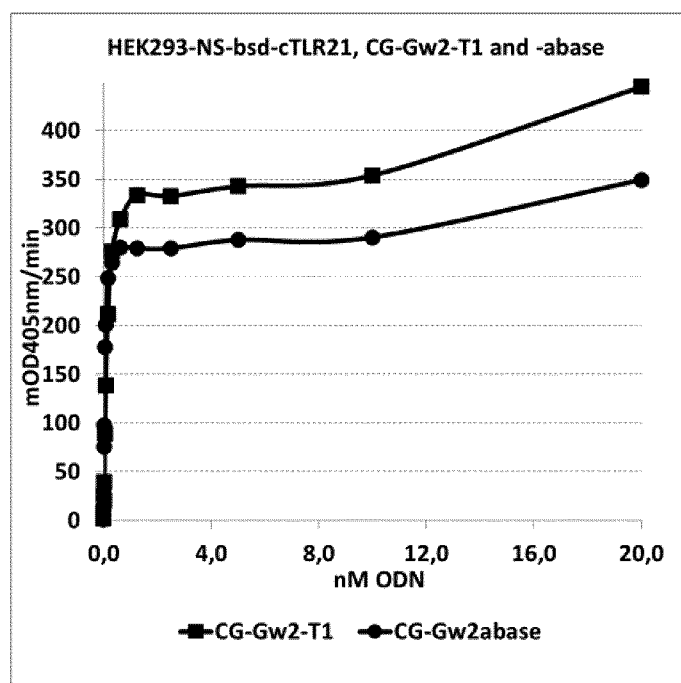


FIG. 42A

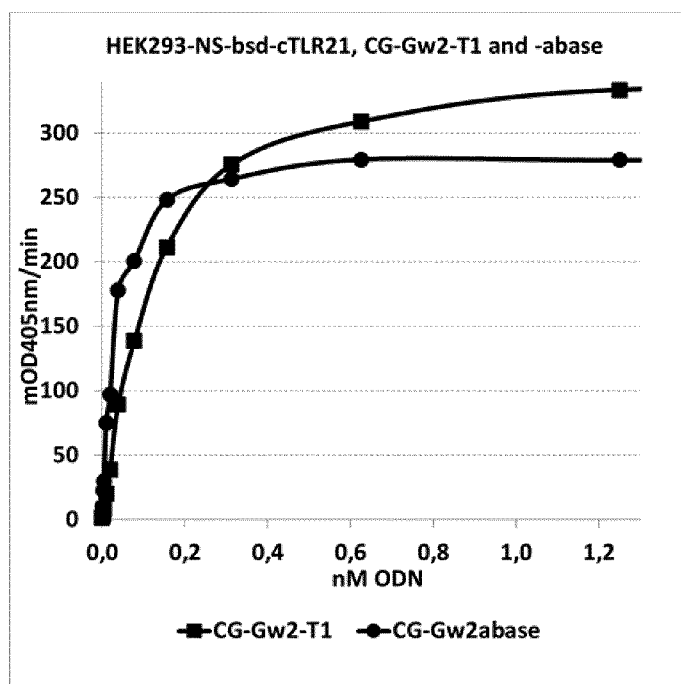
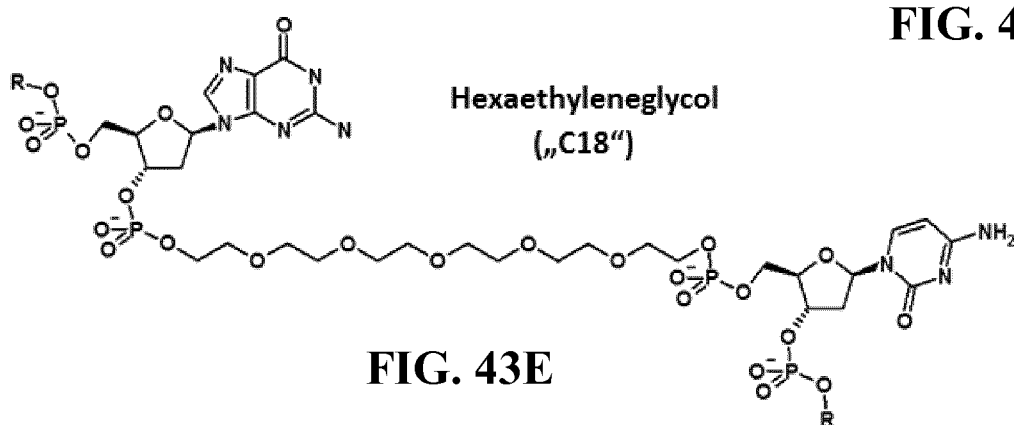
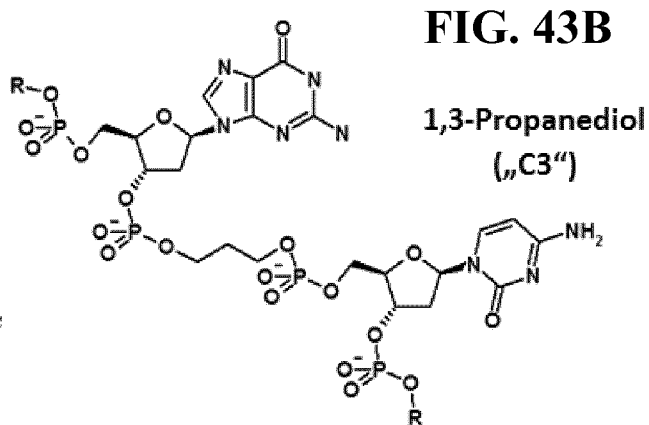
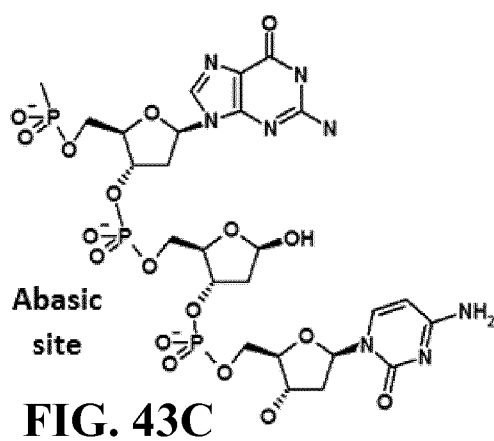
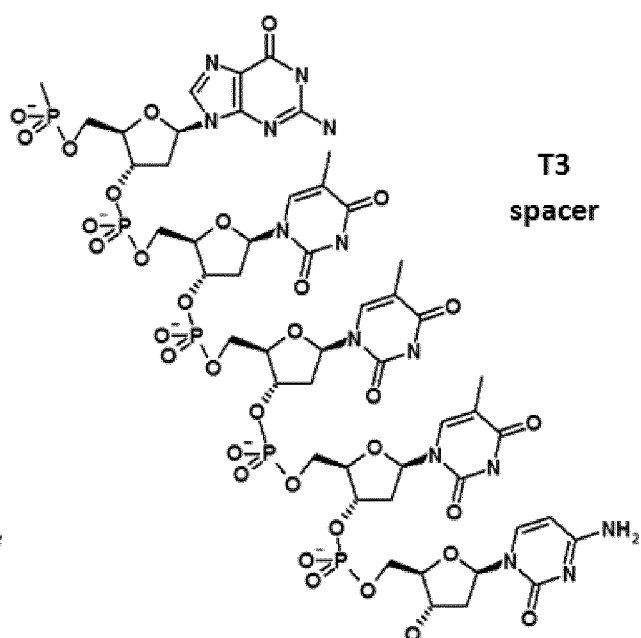
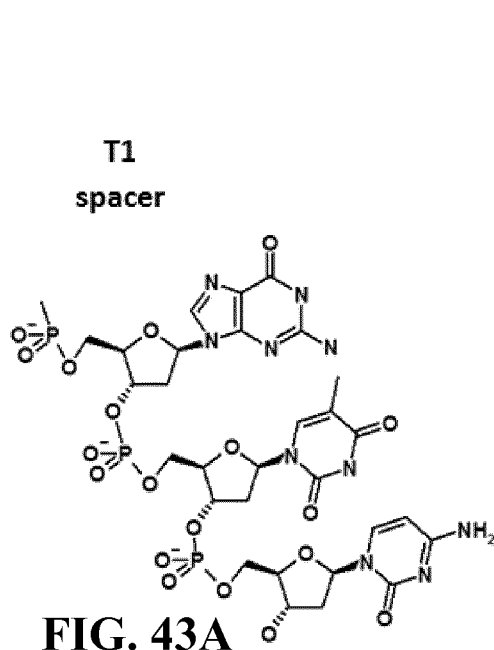


FIG. 42B



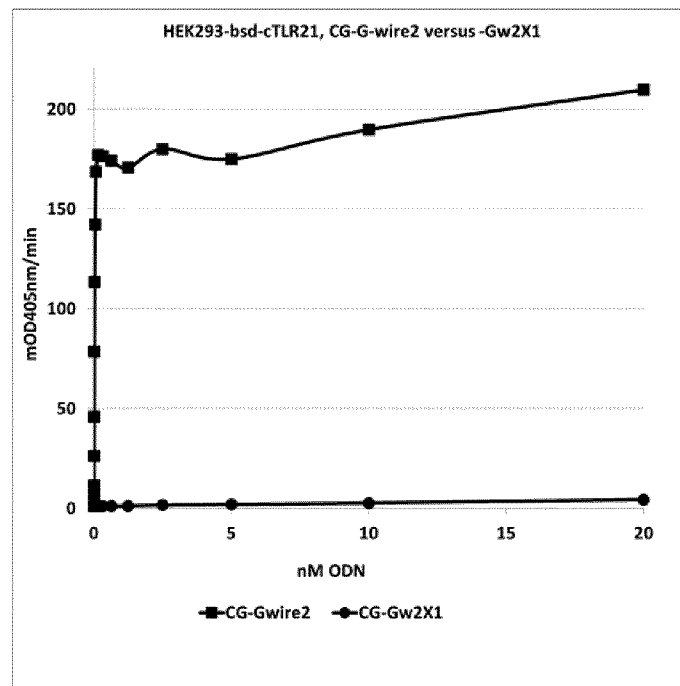


FIG. 44A

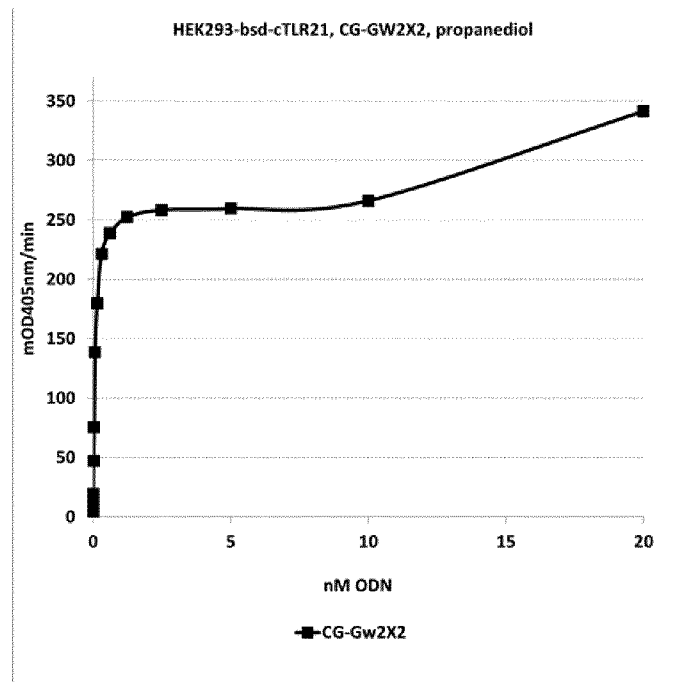


FIG. 44B

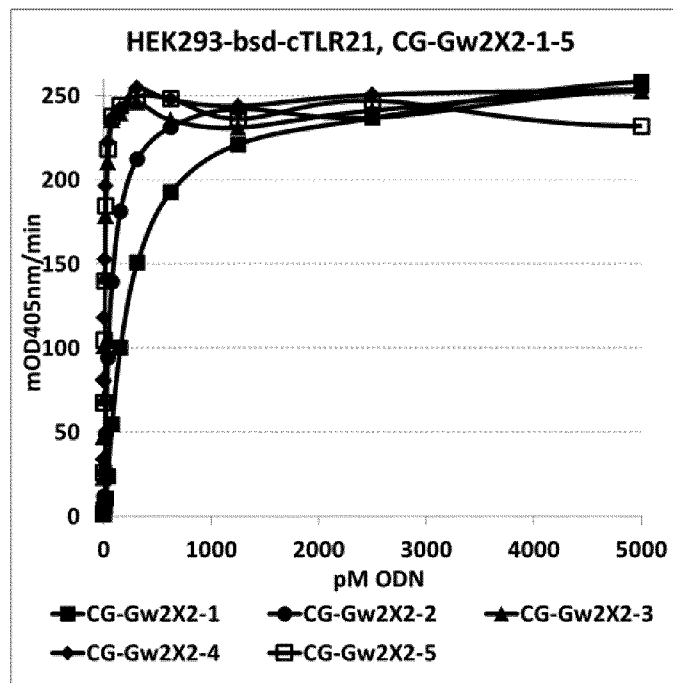


FIG. 45A

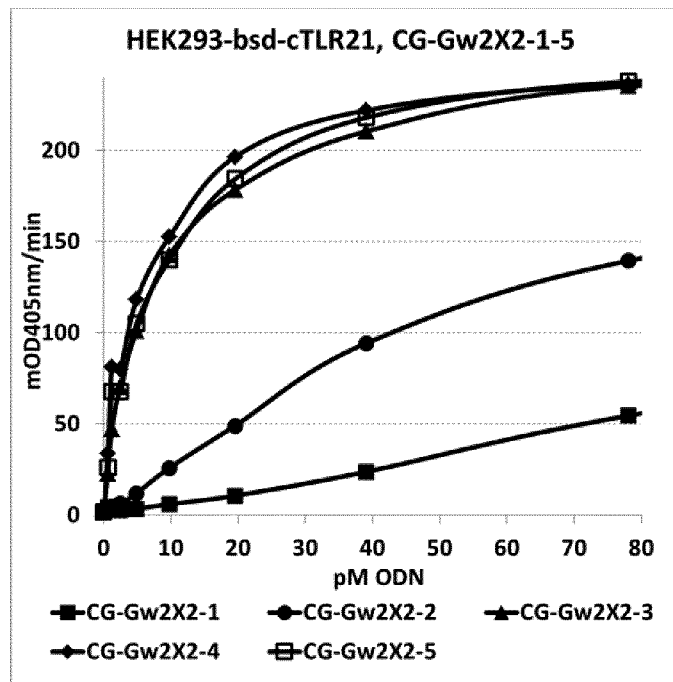


FIG. 45B

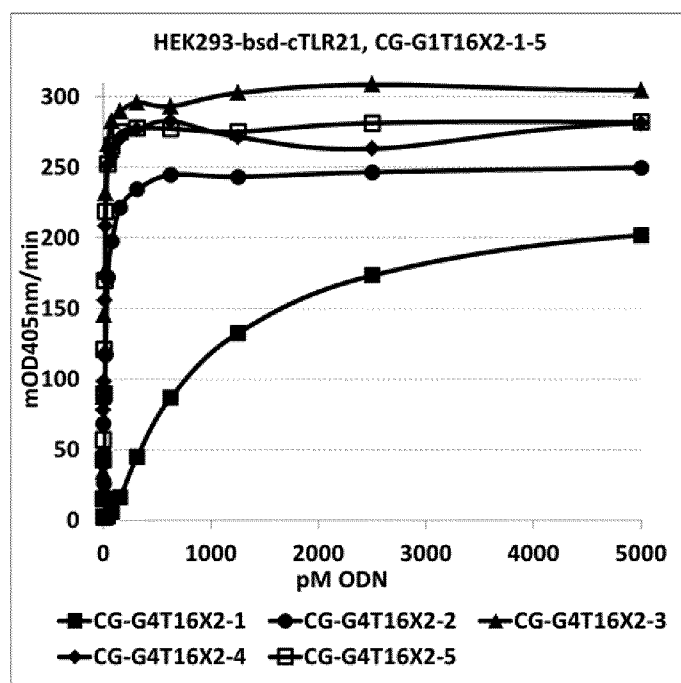


FIG. 46A

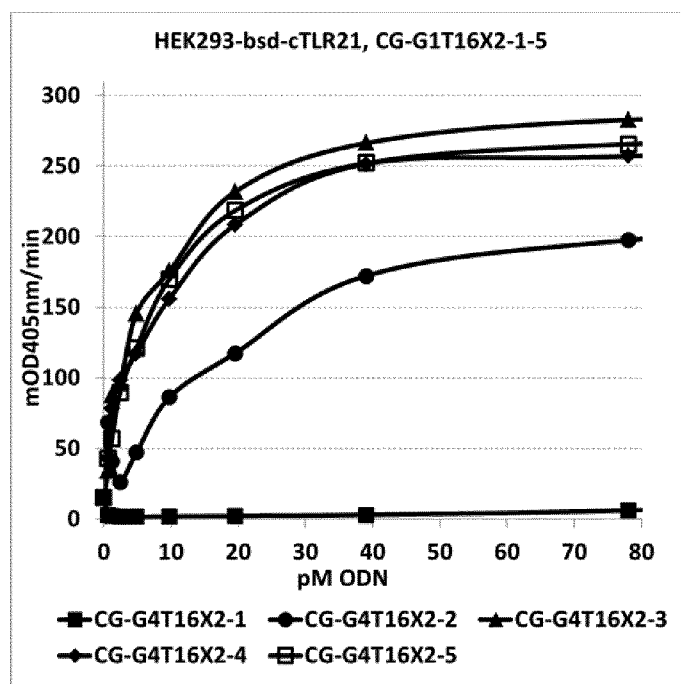


FIG. 46B

Scheme 2:

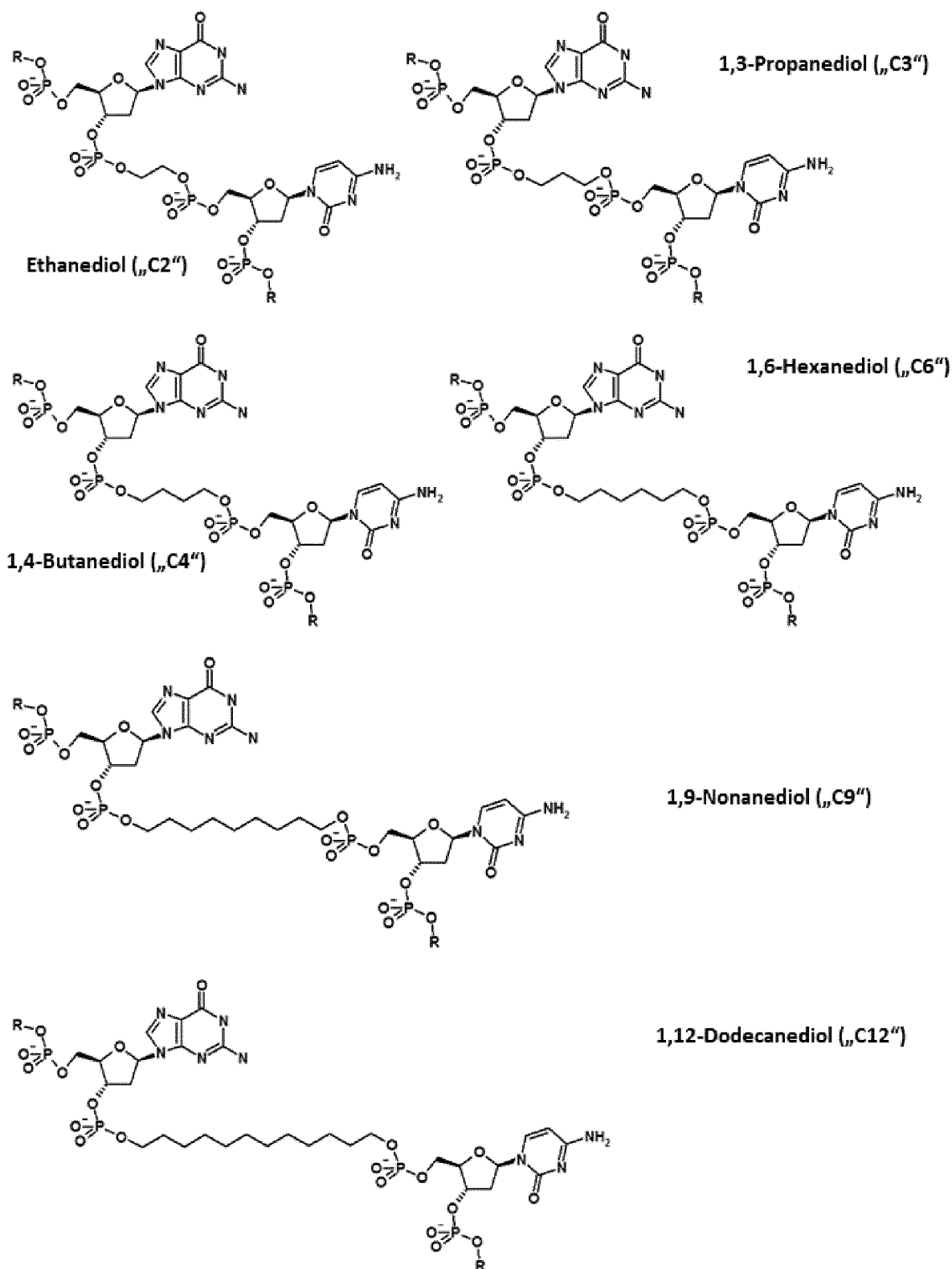


FIG. 47

Scheme 3:

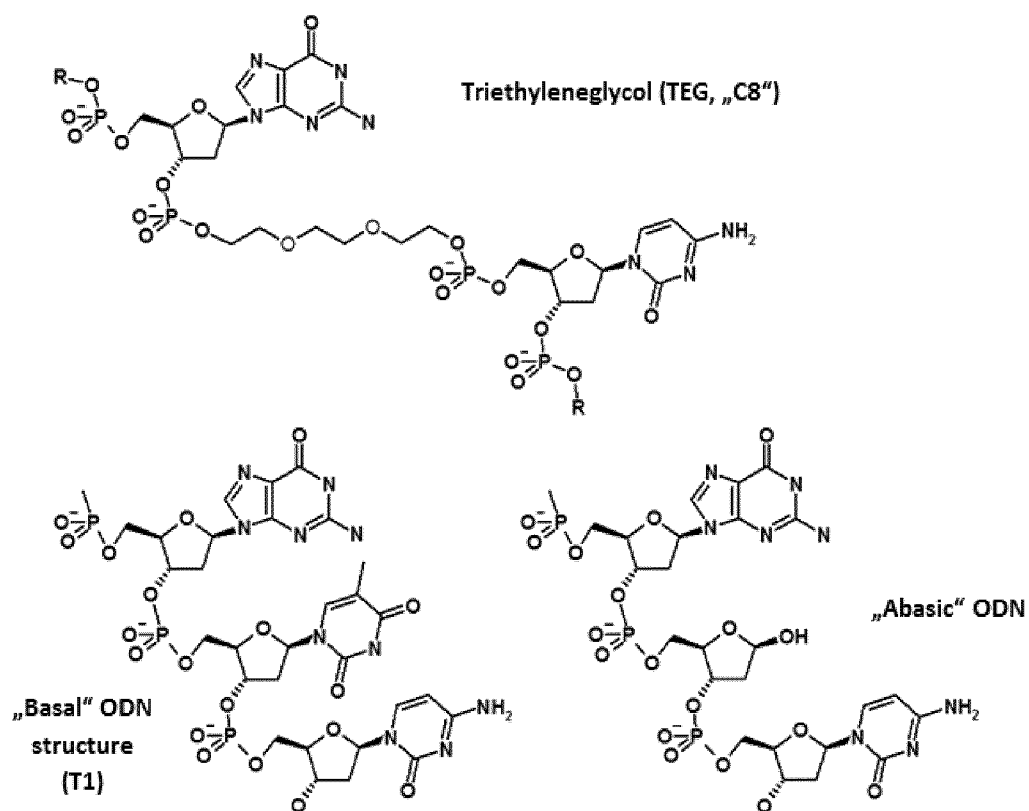


FIG. 48

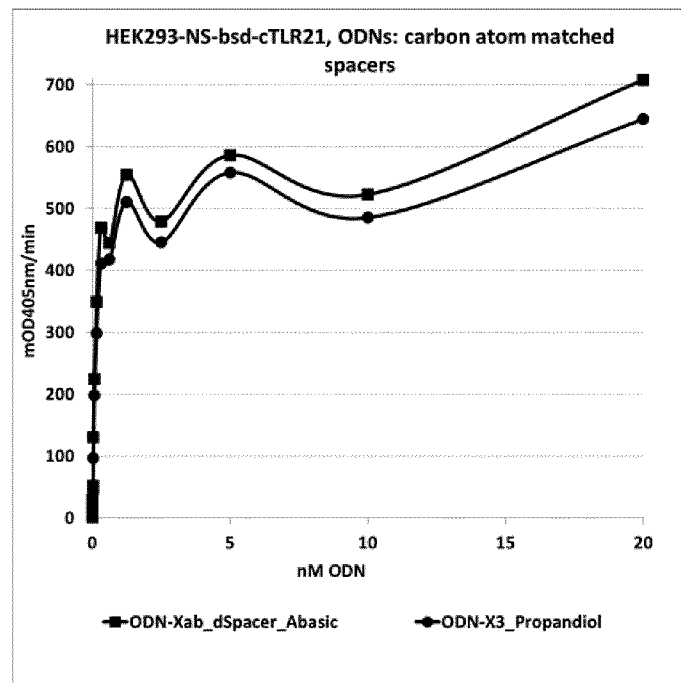


FIG. 49A

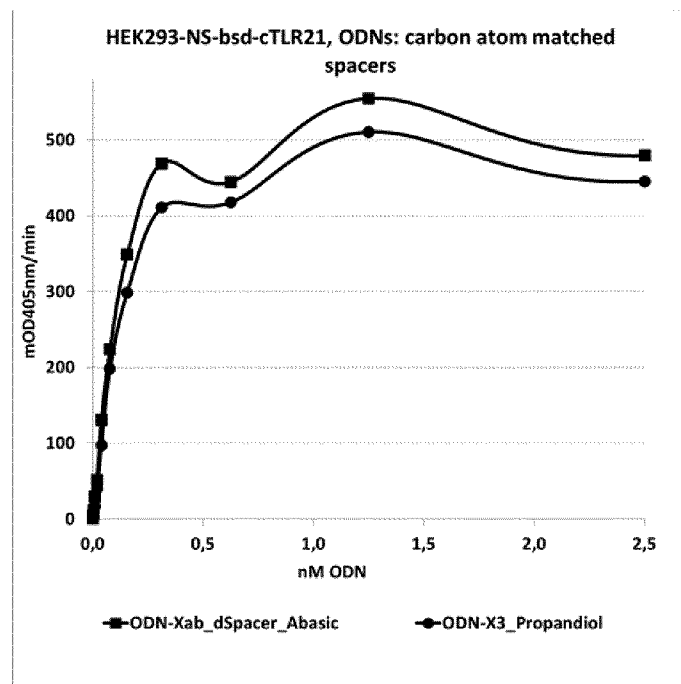


FIG. 49B

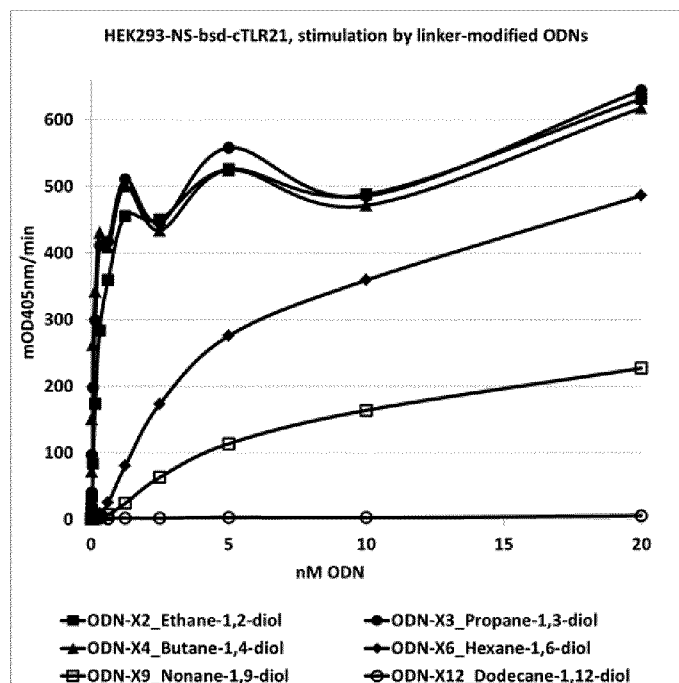


FIG. 50A

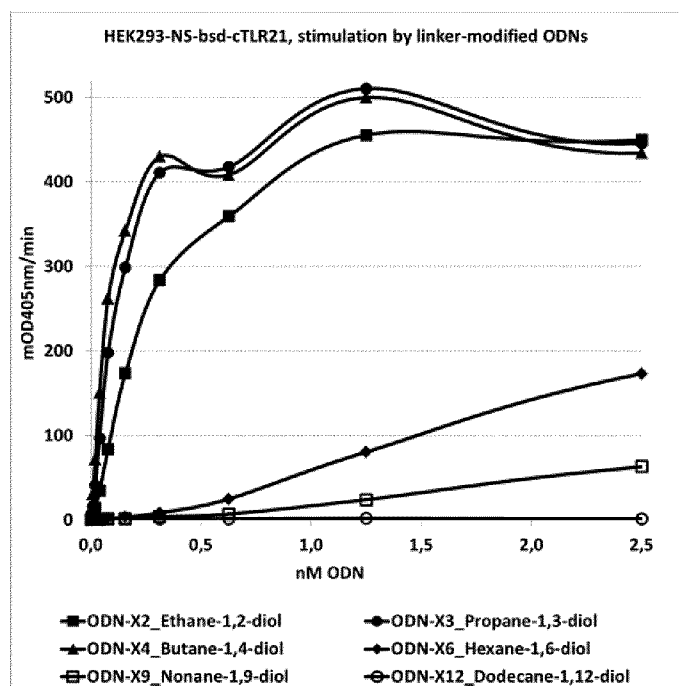


FIG. 50B

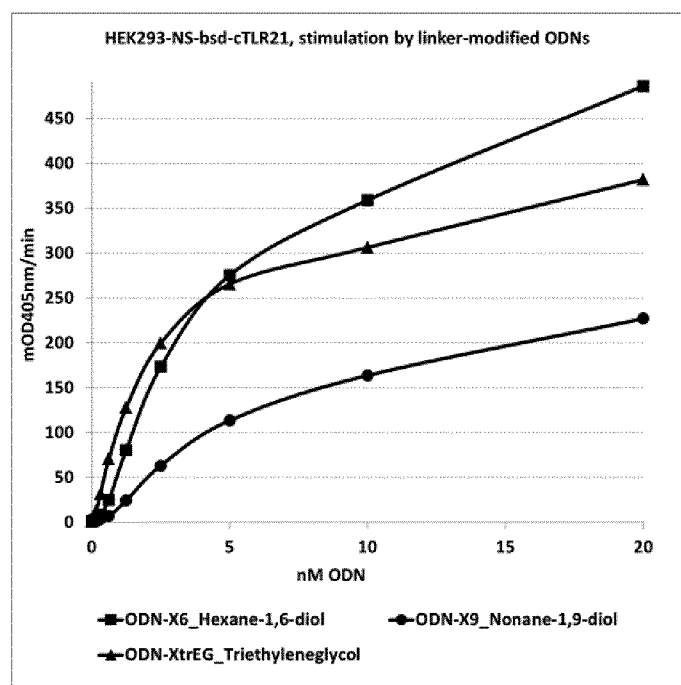


FIG. 51

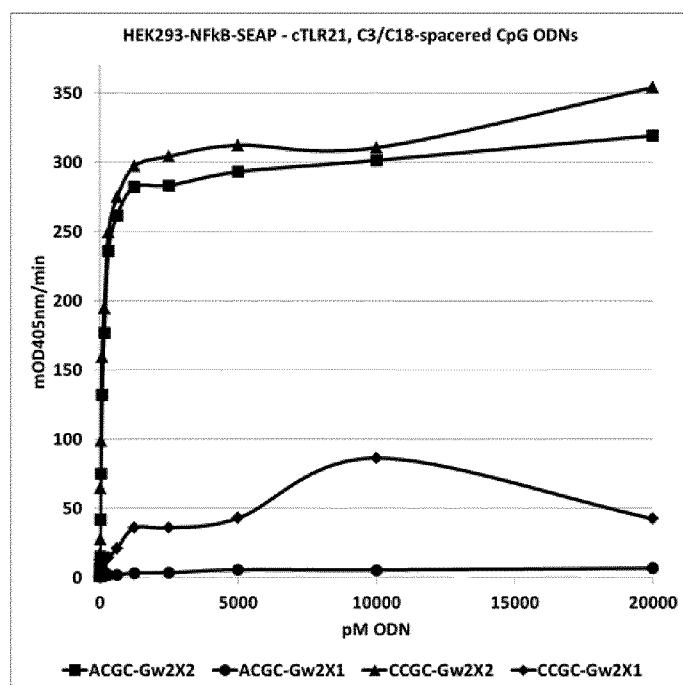


FIG. 52A

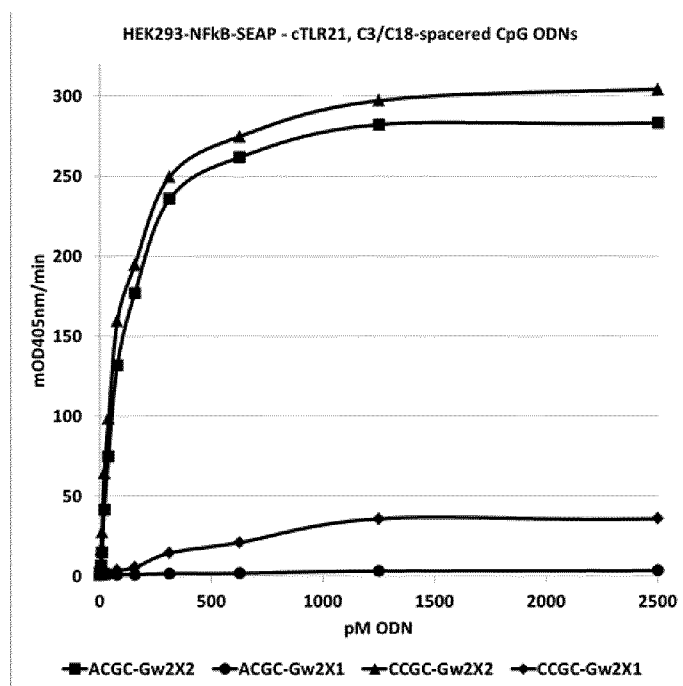


FIG. 52B

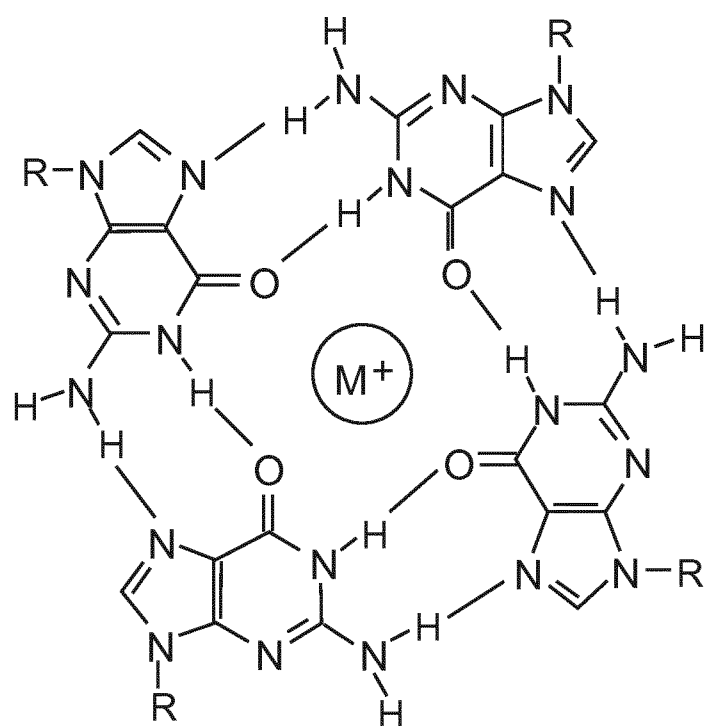


Fig. 23A

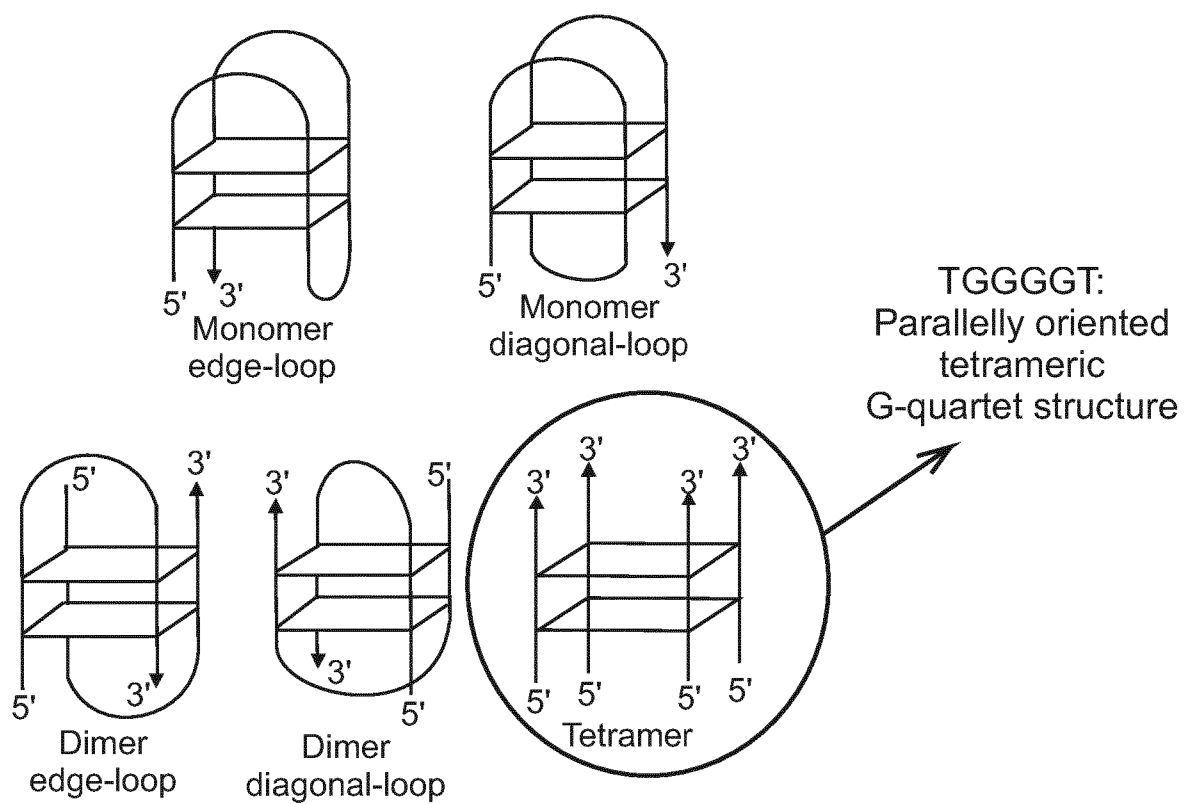


Fig. 23B

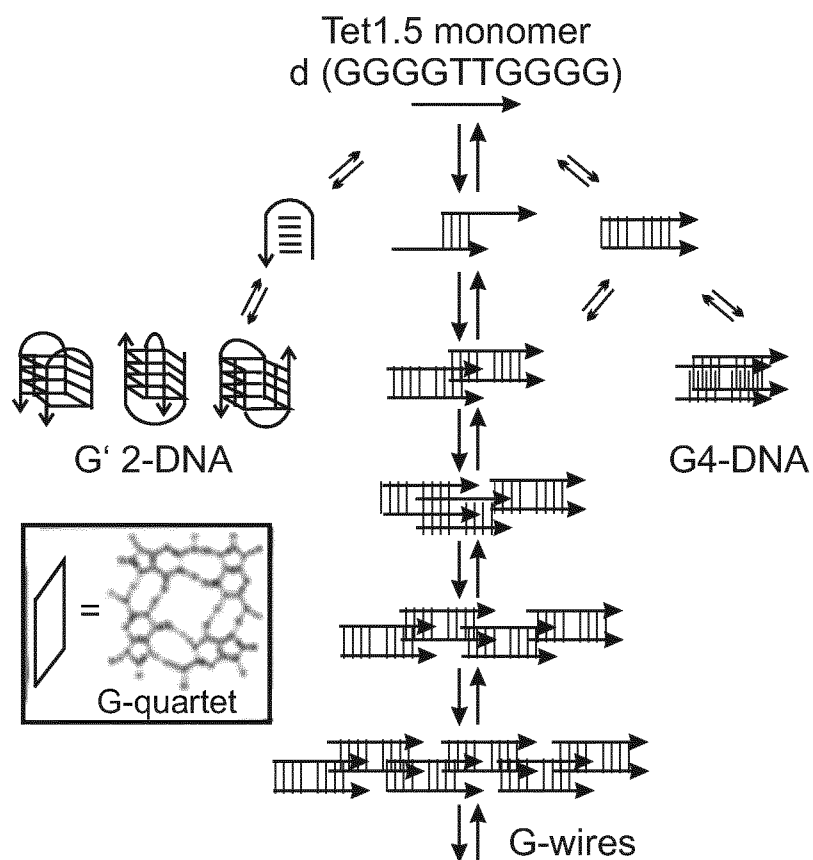
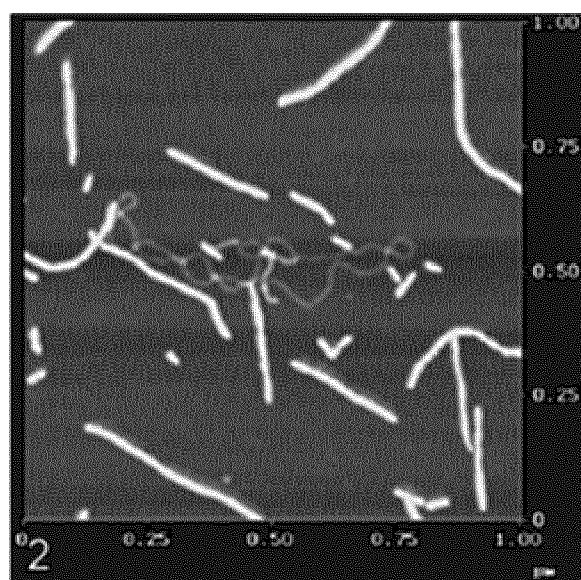


Fig. 23C



GGGGTTGGGG:
G-wire with the
potential to
„frayed“ G-wire

Fig. 23D

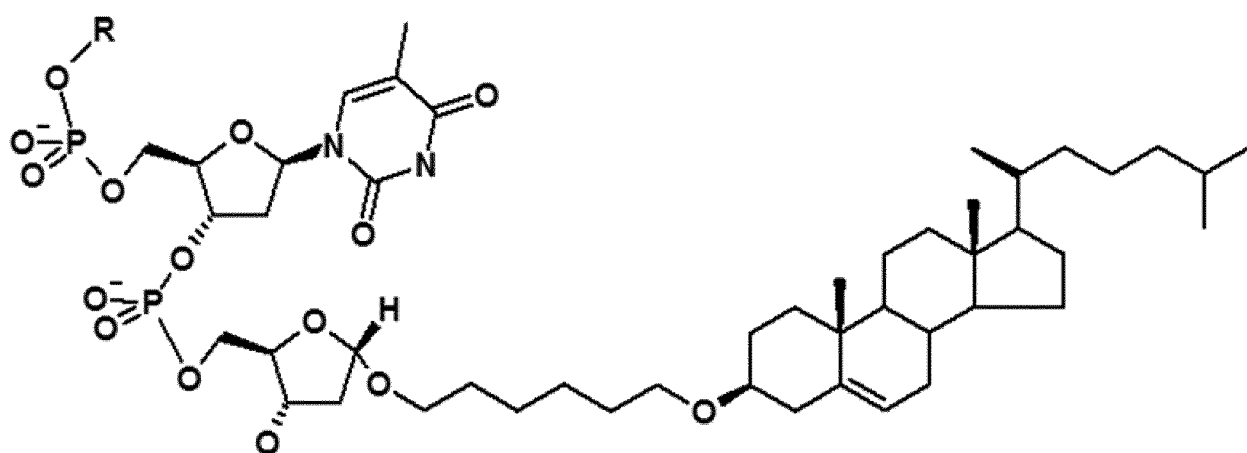


FIG. 55

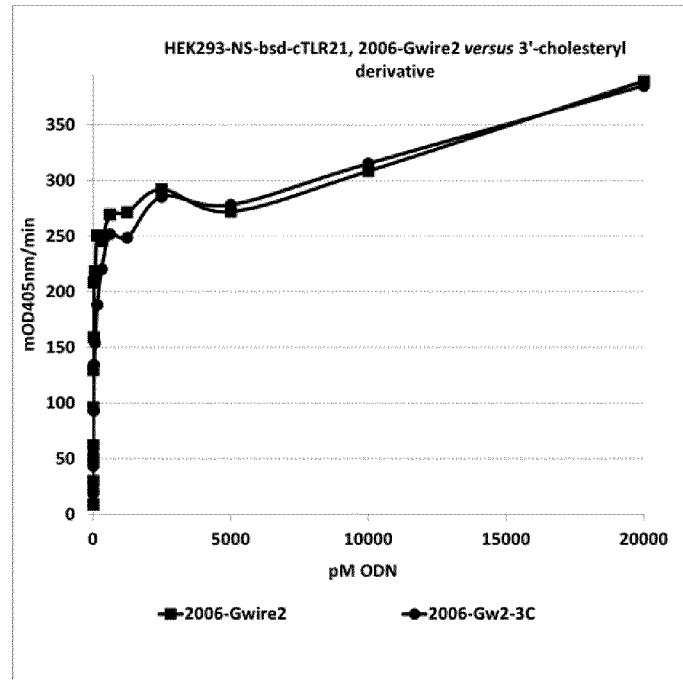


FIG. 56A

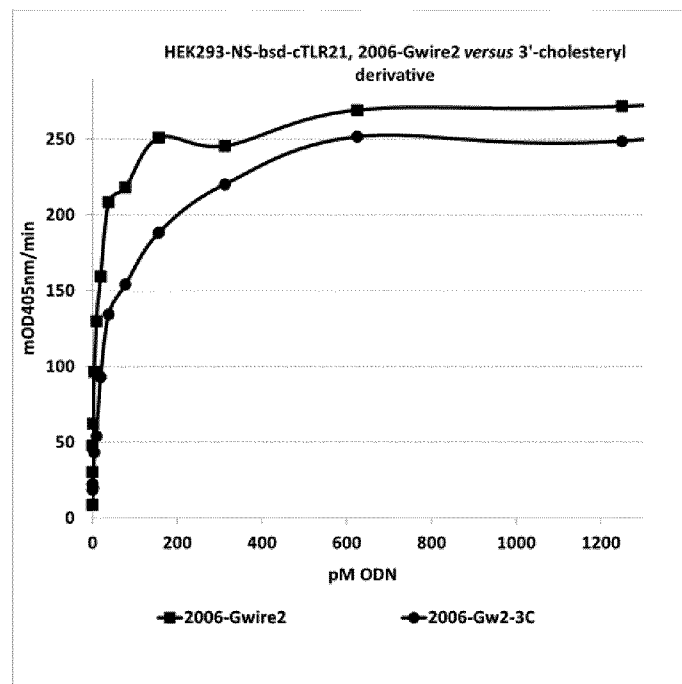


FIG. 56B

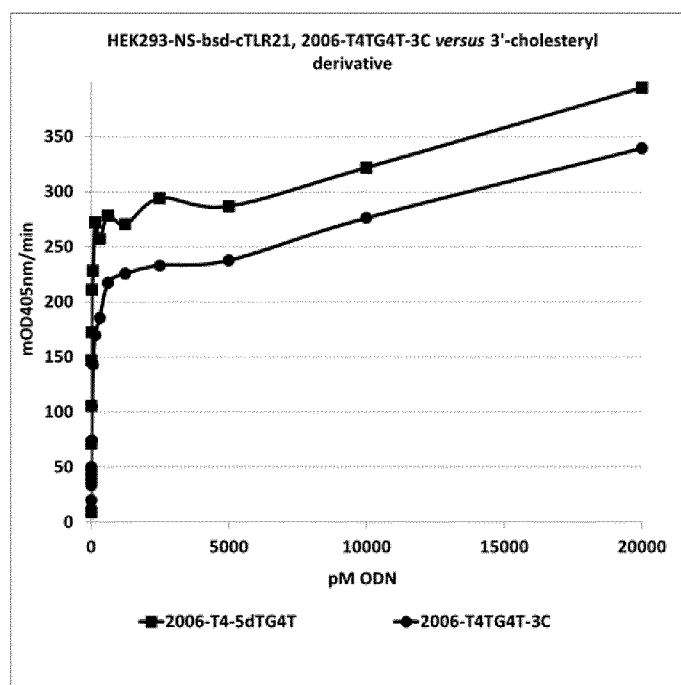


FIG. 57A

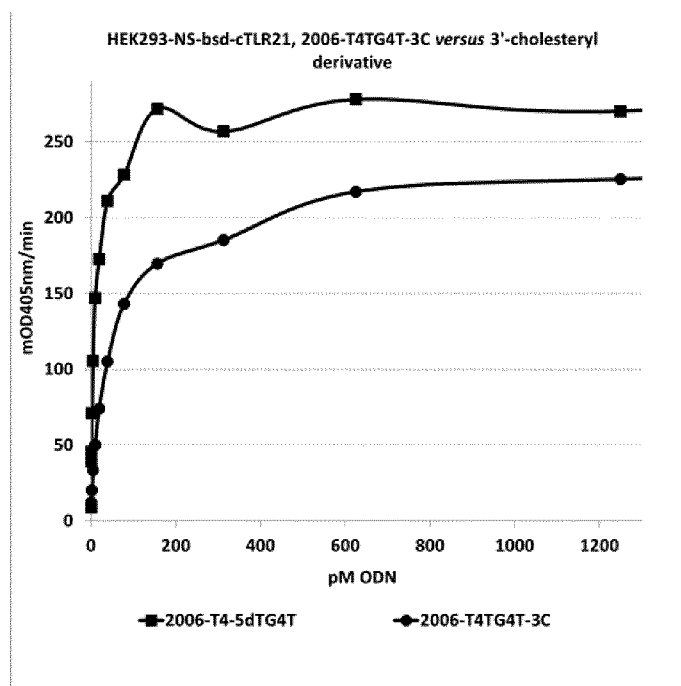
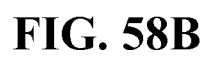


FIG. 57B



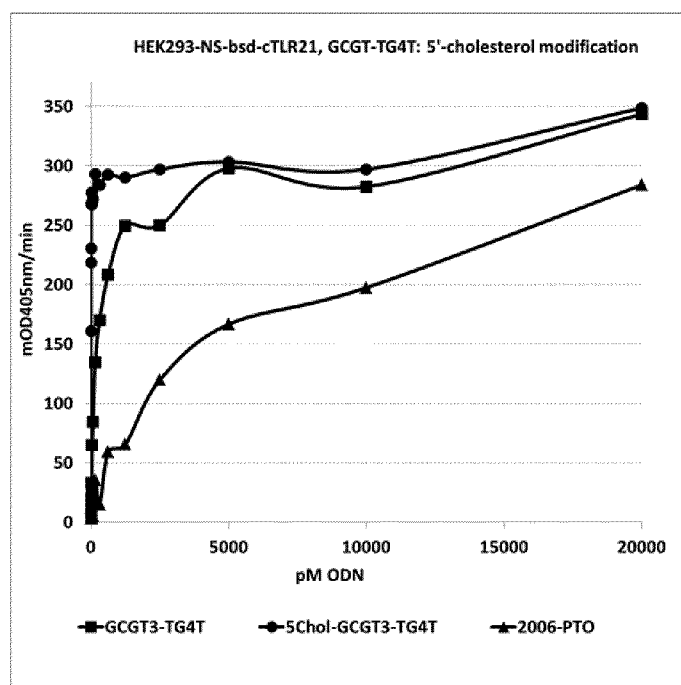


FIG. 59A

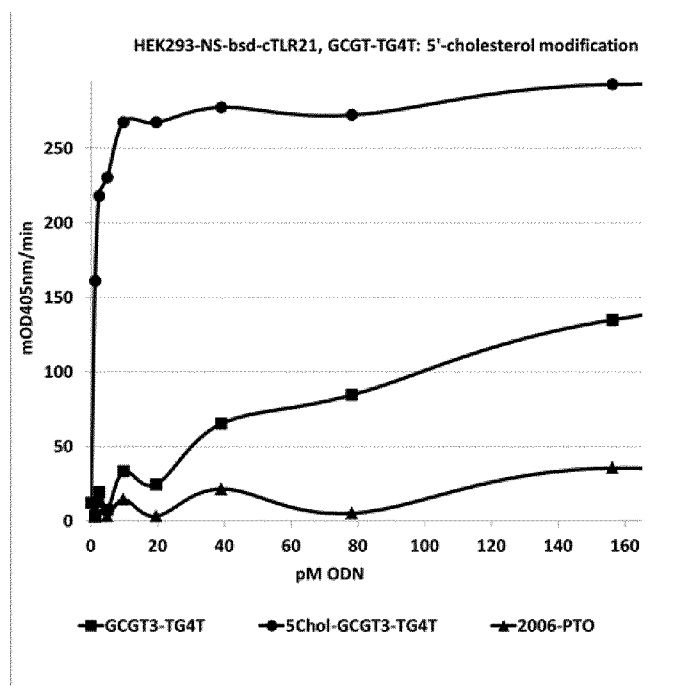


FIG. 59B

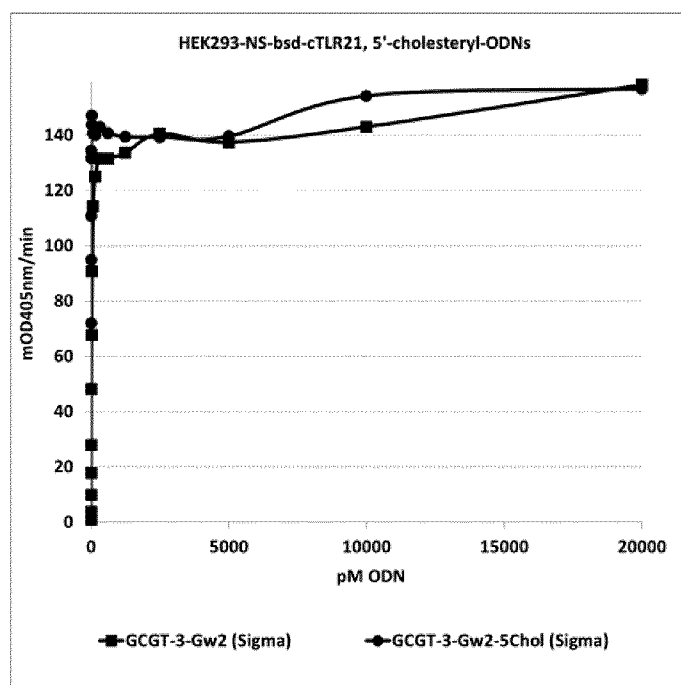


FIG. 60A

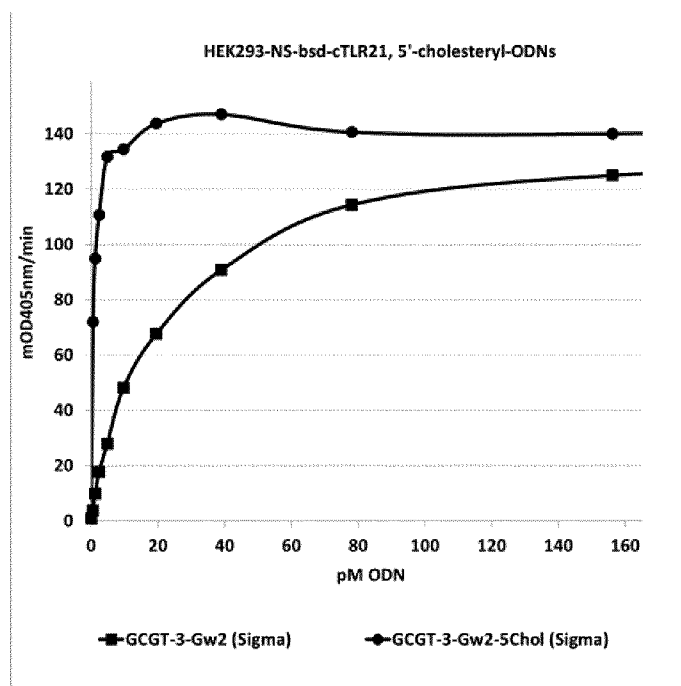


FIG. 60B

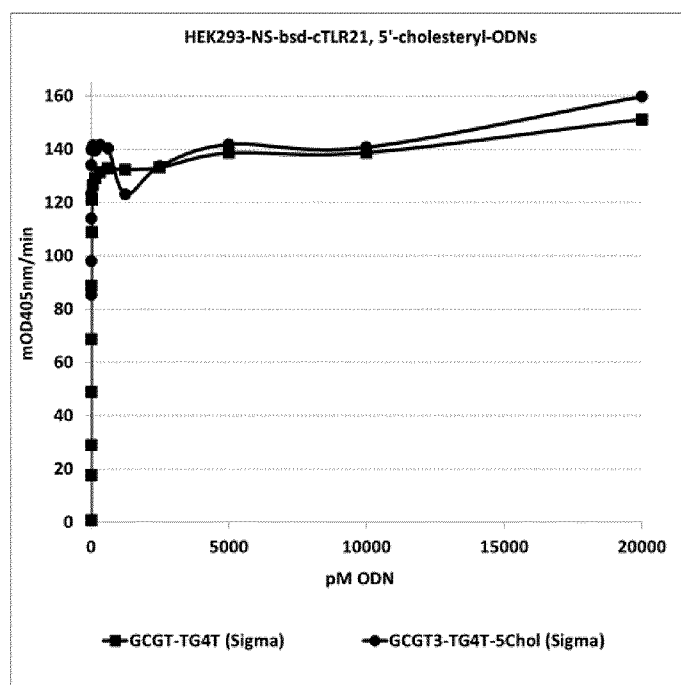


FIG. 61A

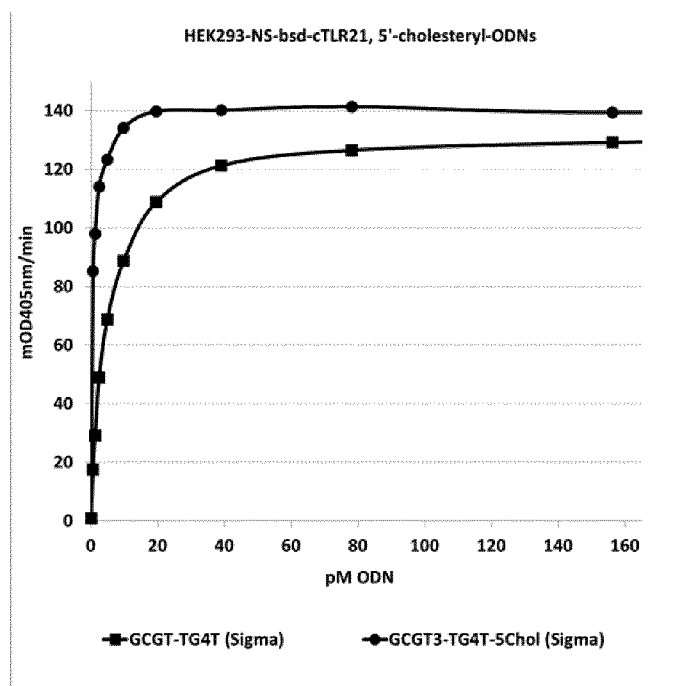


FIG. 61B

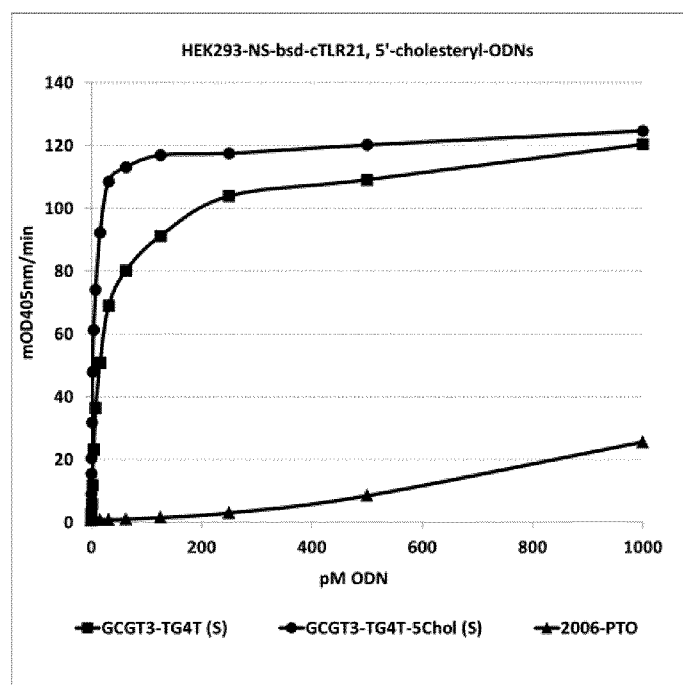


FIG. 62A

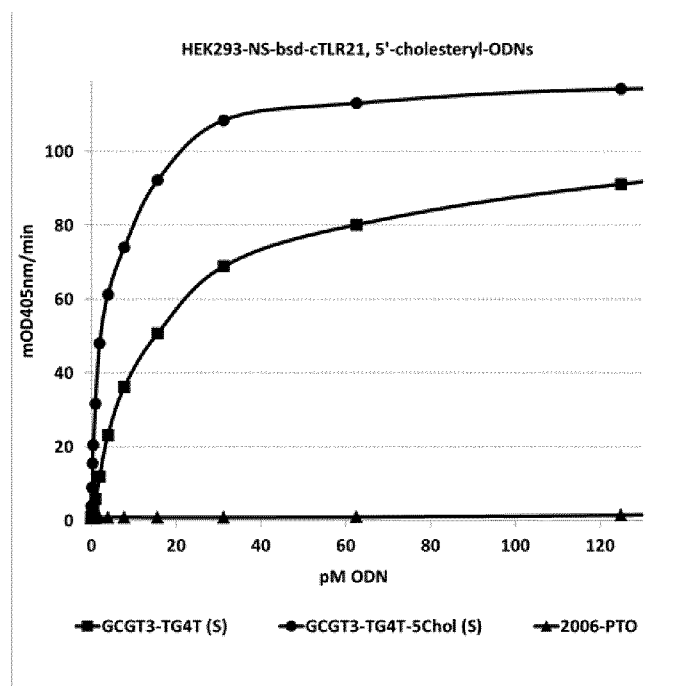


FIG. 62B

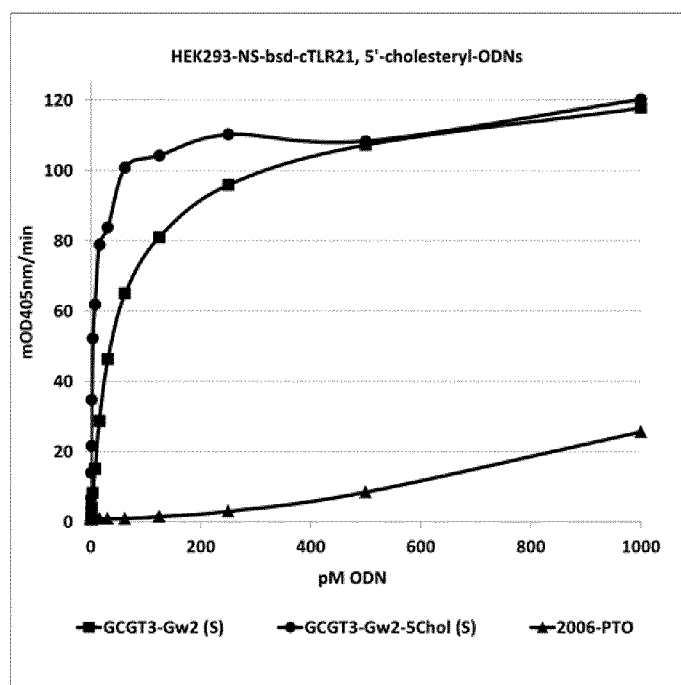


FIG. 63A

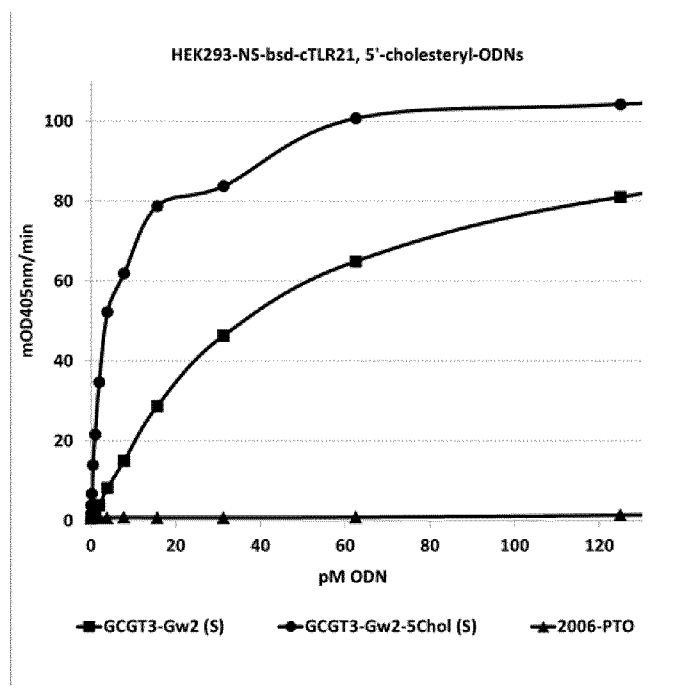


FIG. 63B

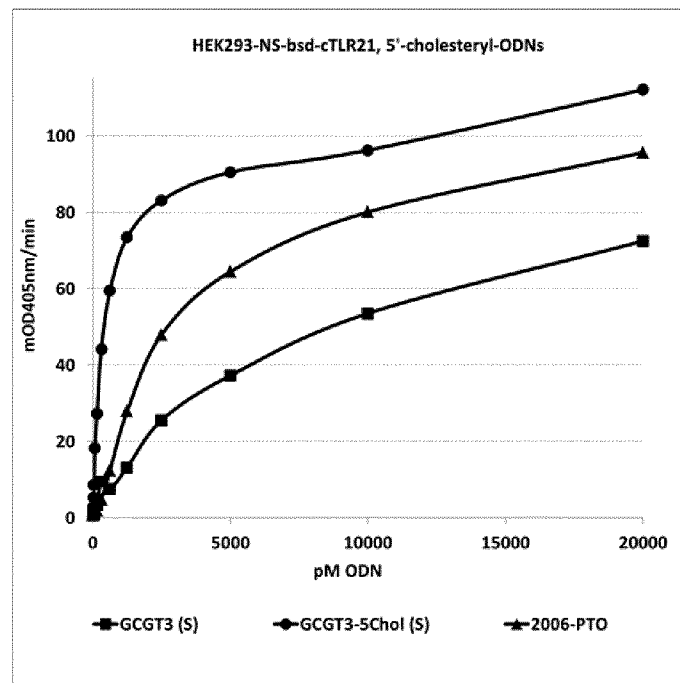


FIG. 64

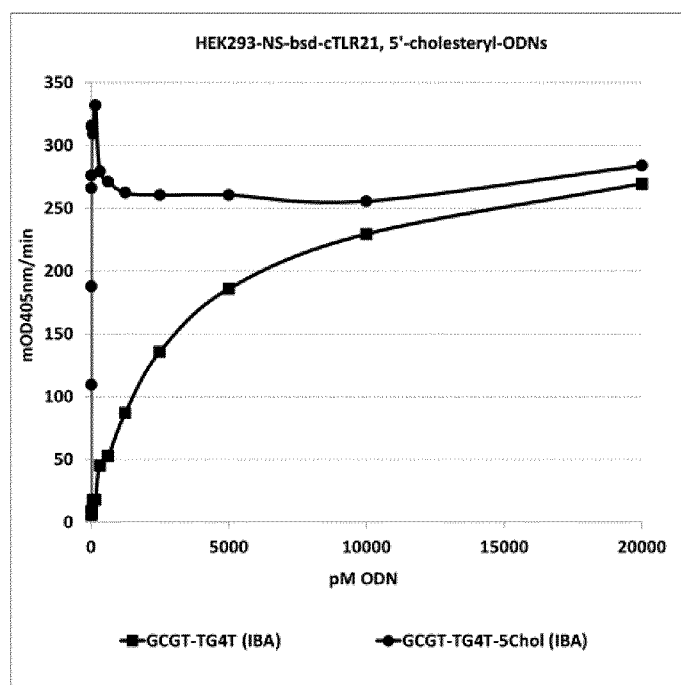


FIG. 65A

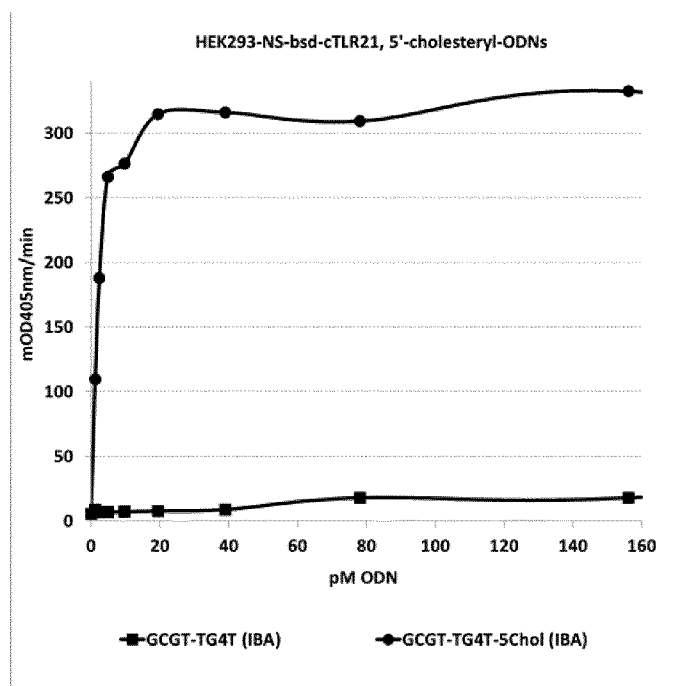


FIG. 65B

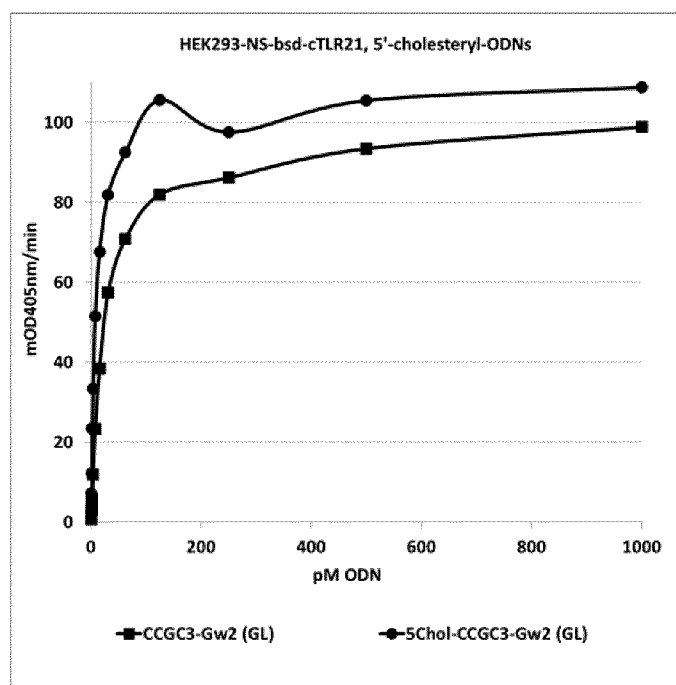


FIG. 66A

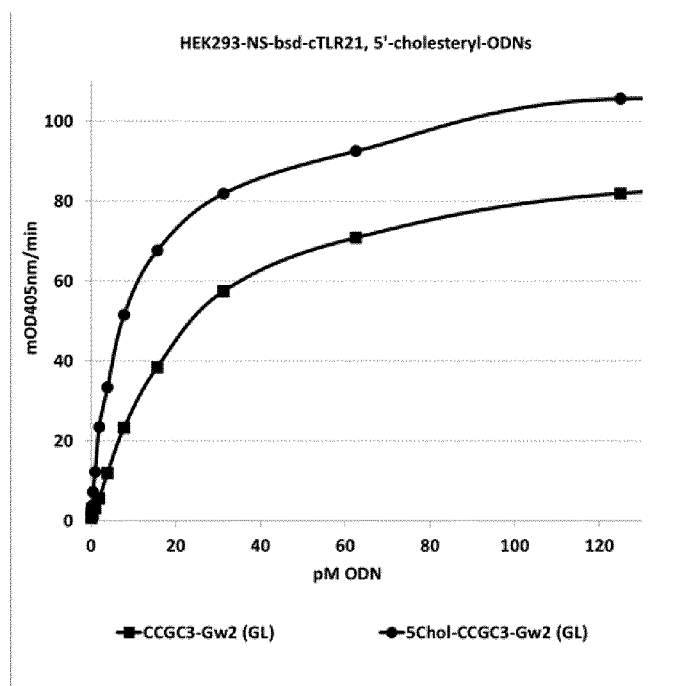
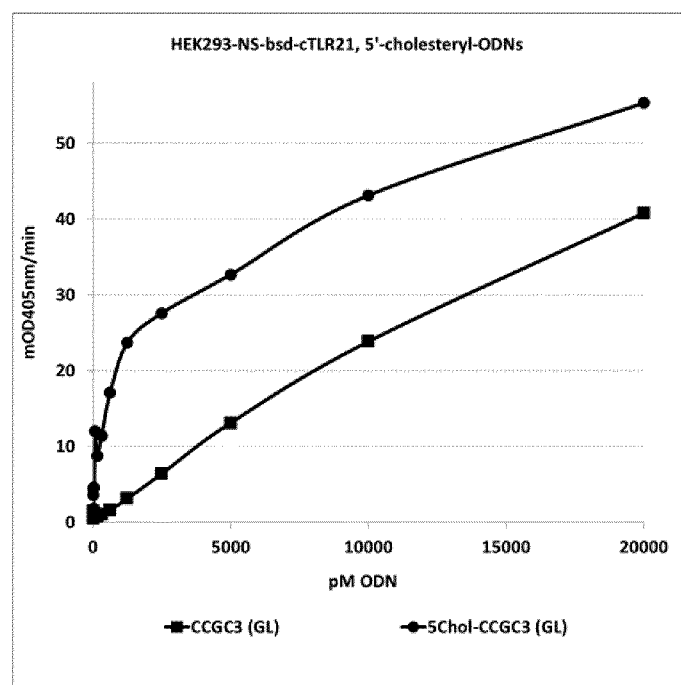


FIG. 66B

**FIG. 67**

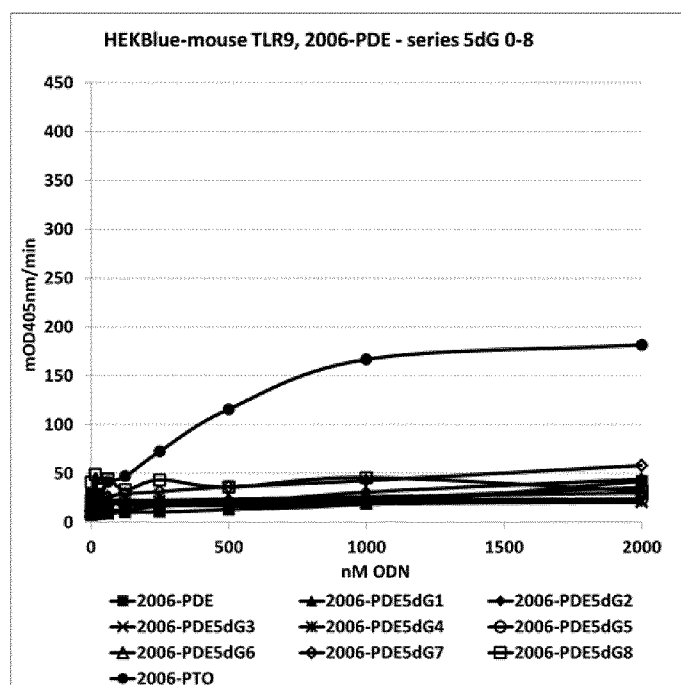


FIG. 68A

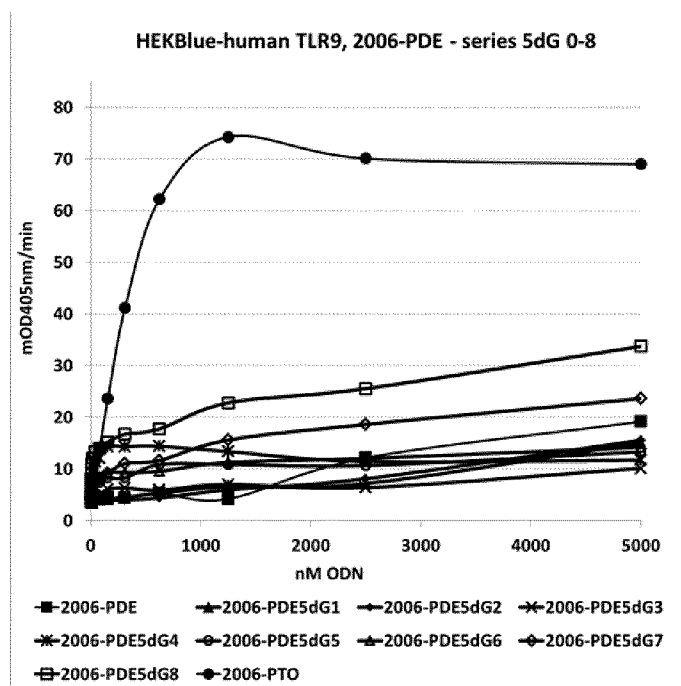


FIG. 68B

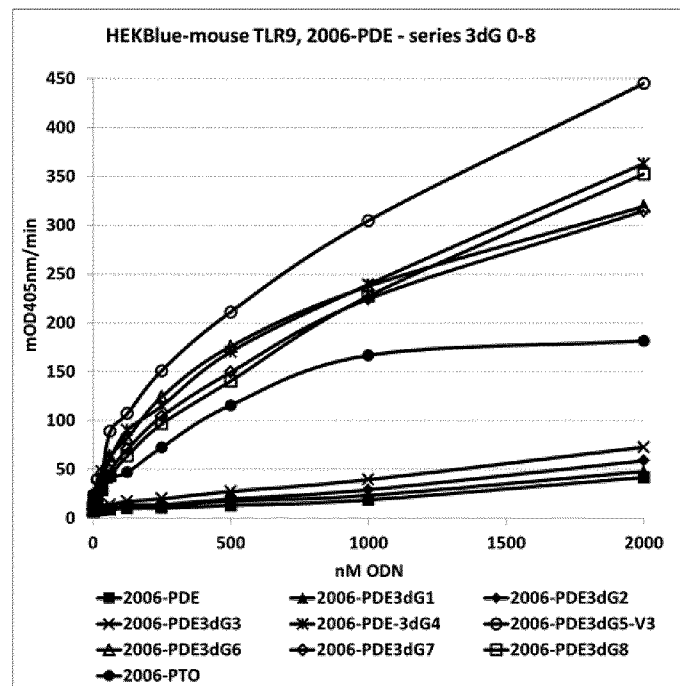


FIG. 69A

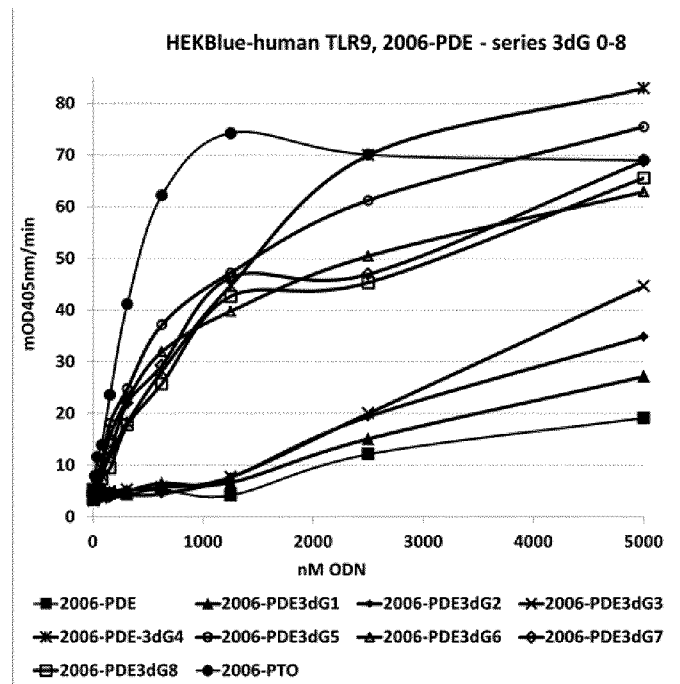
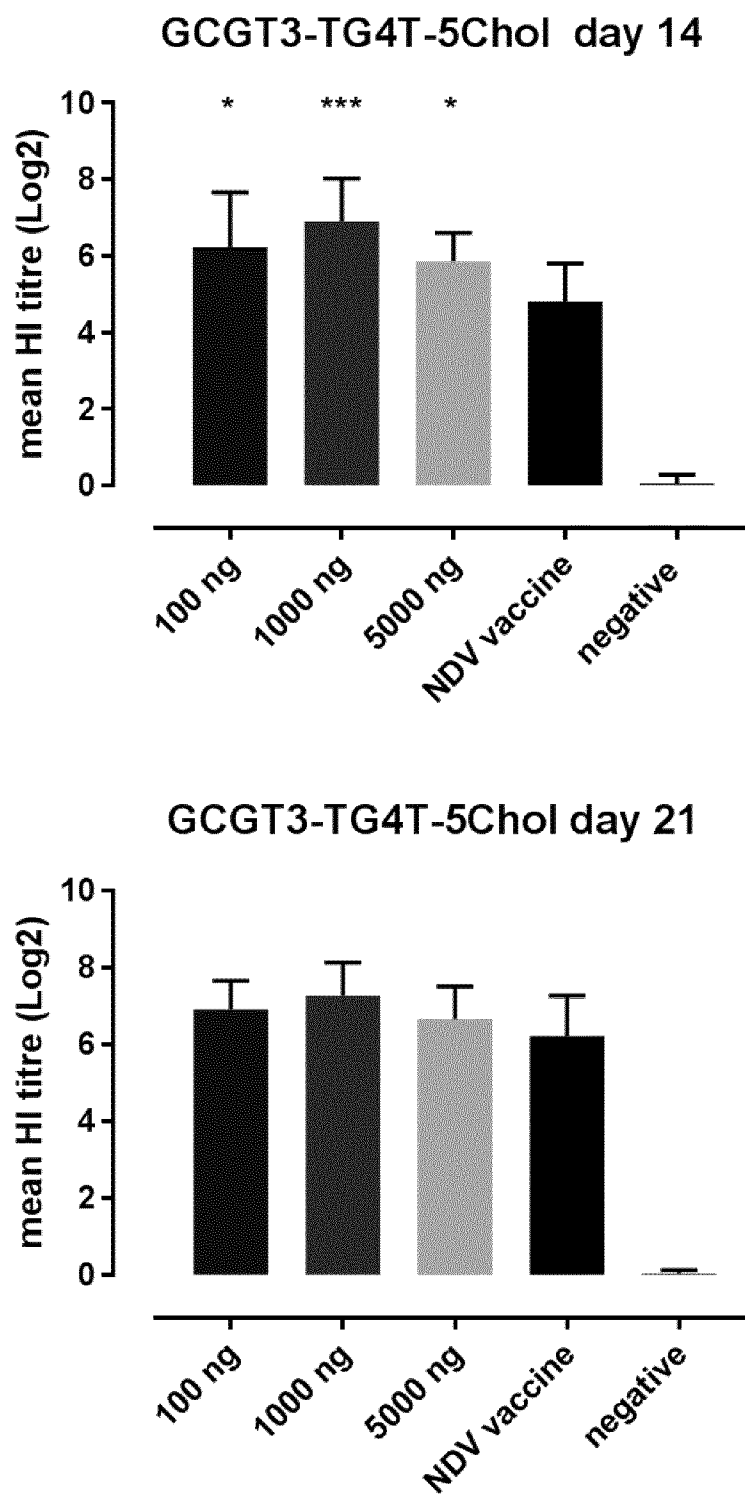
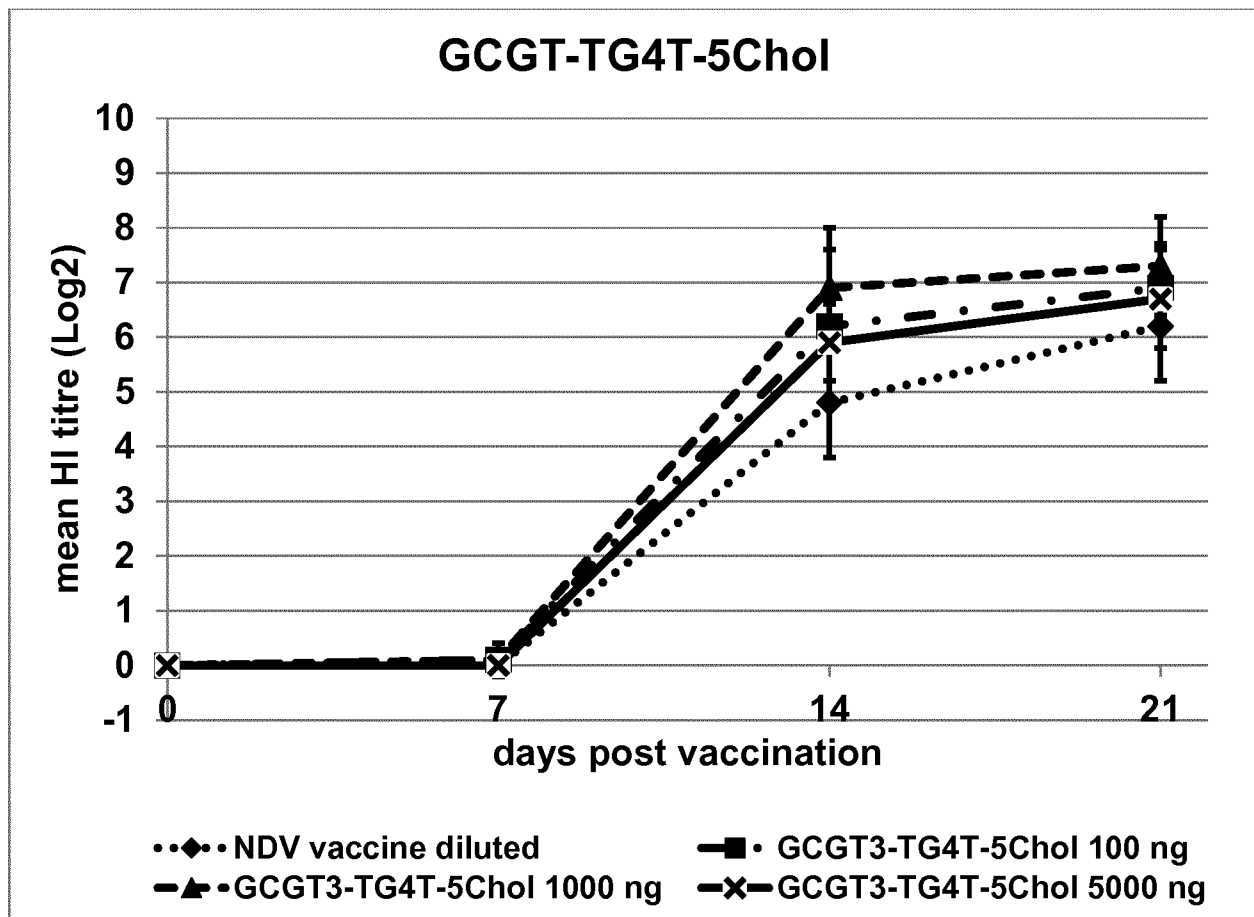
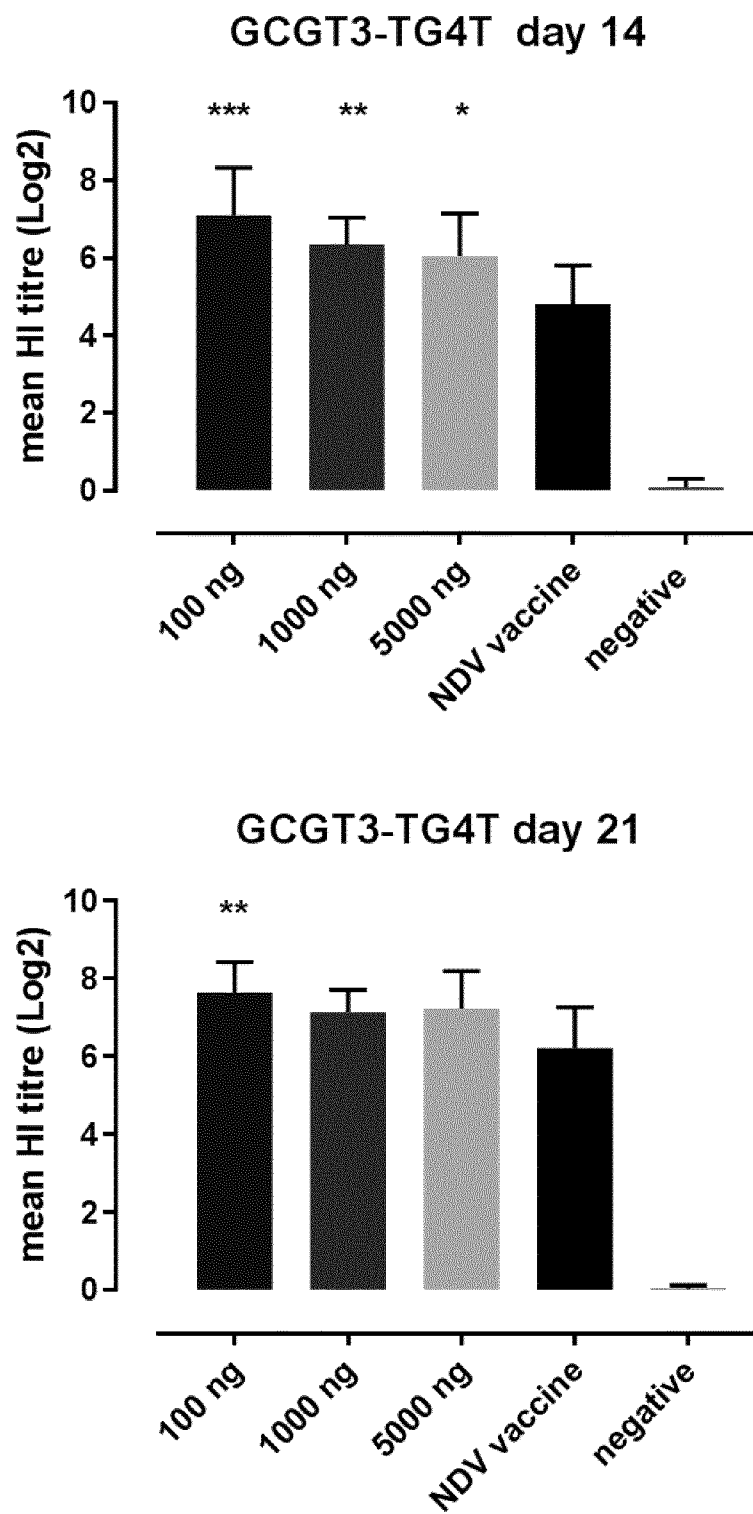
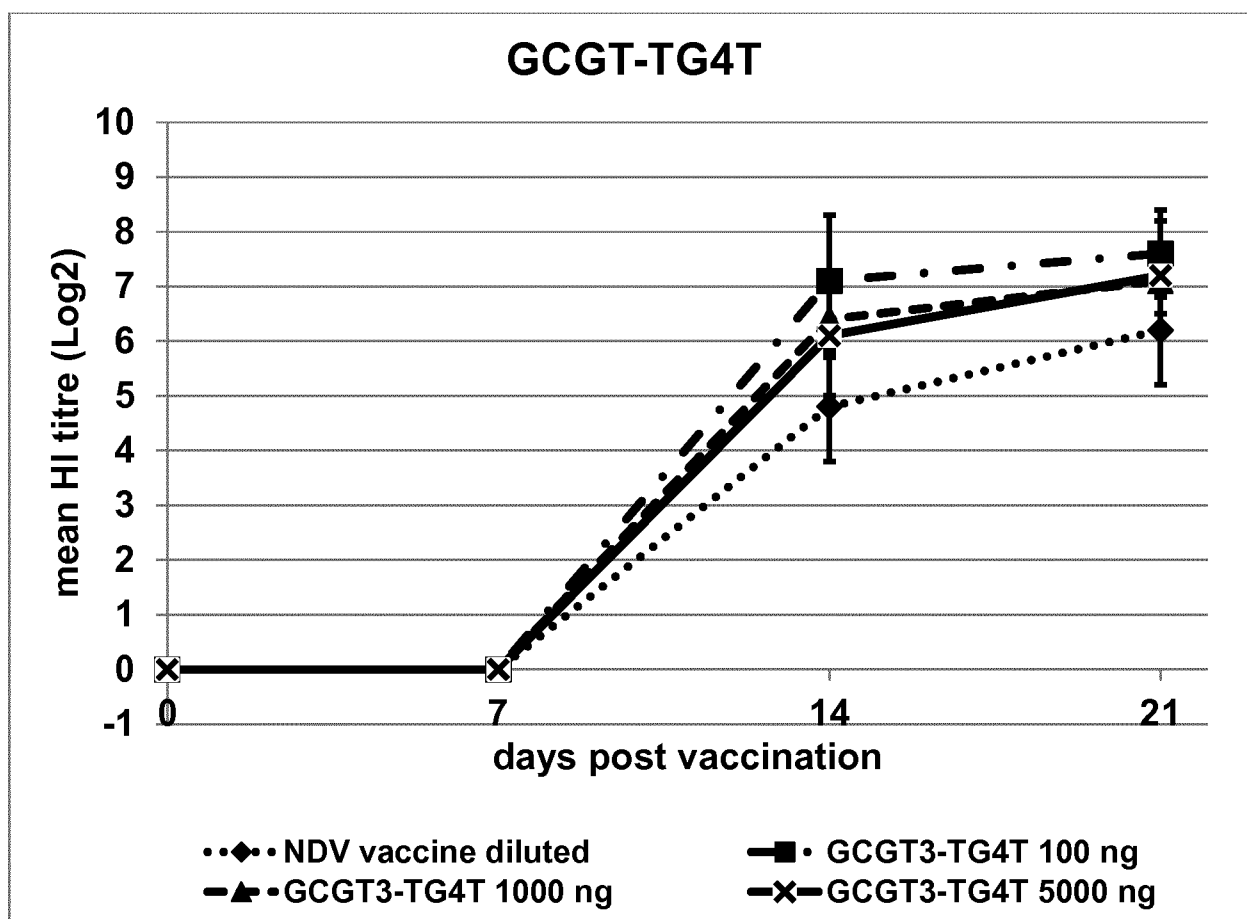


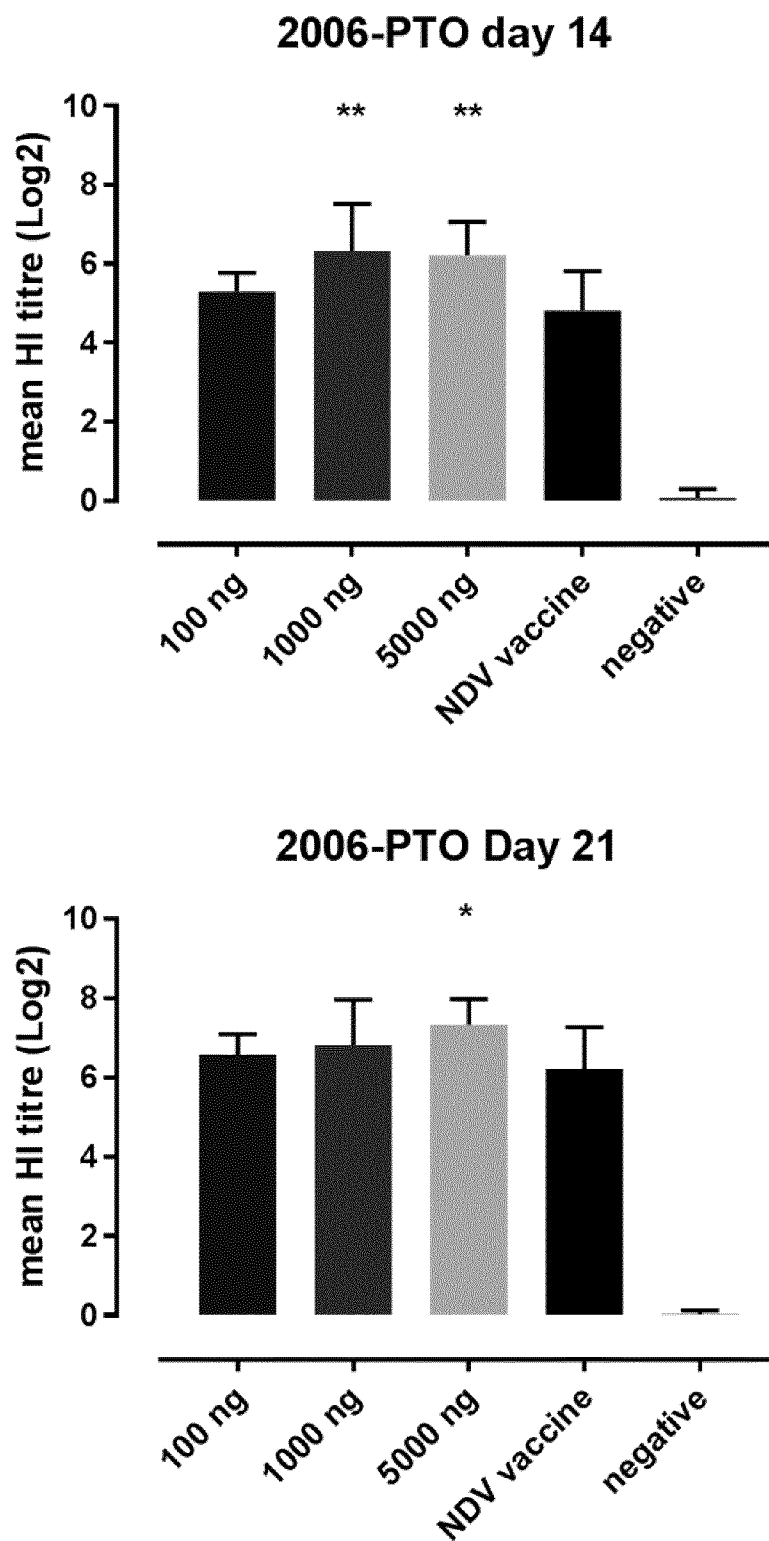
FIG. 69B

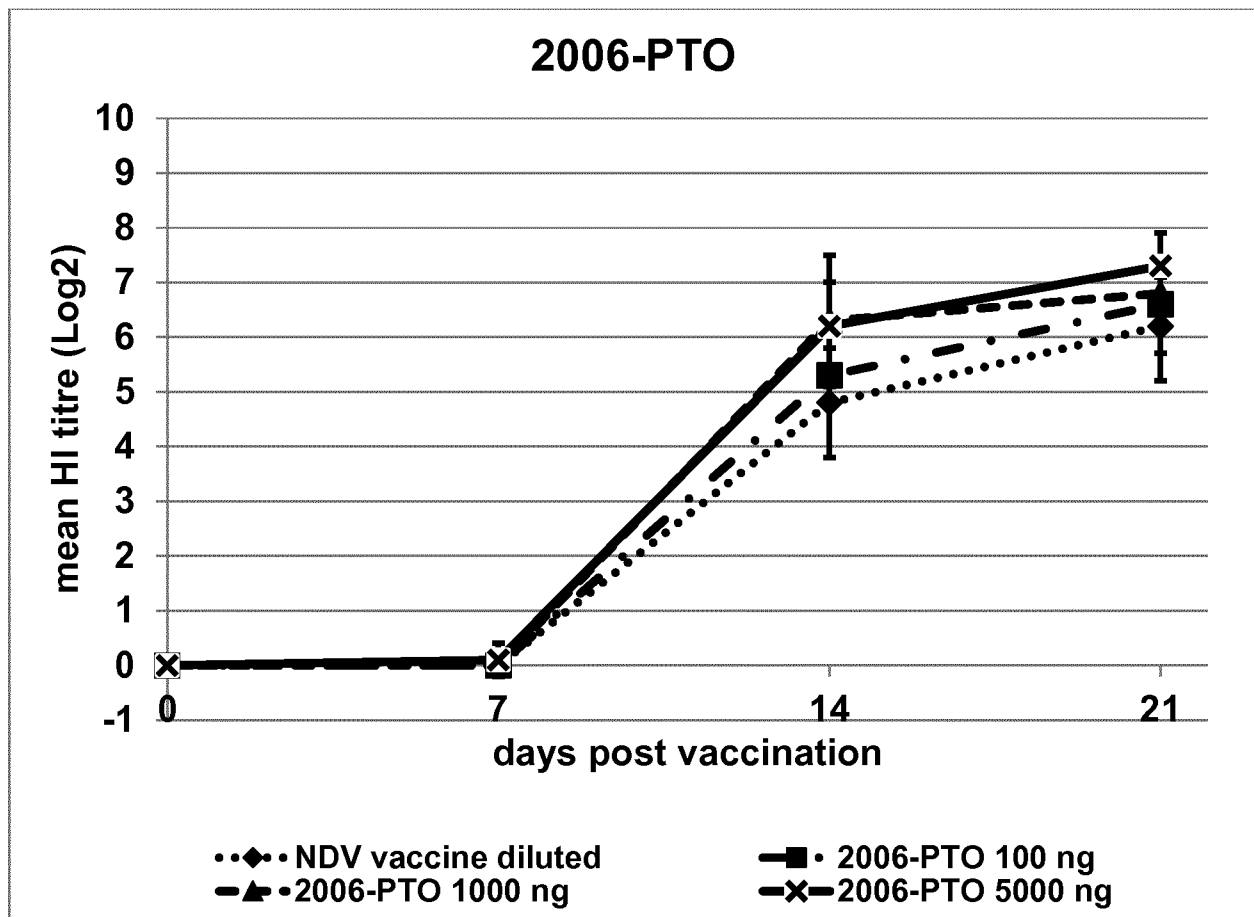
**FIG. 70**

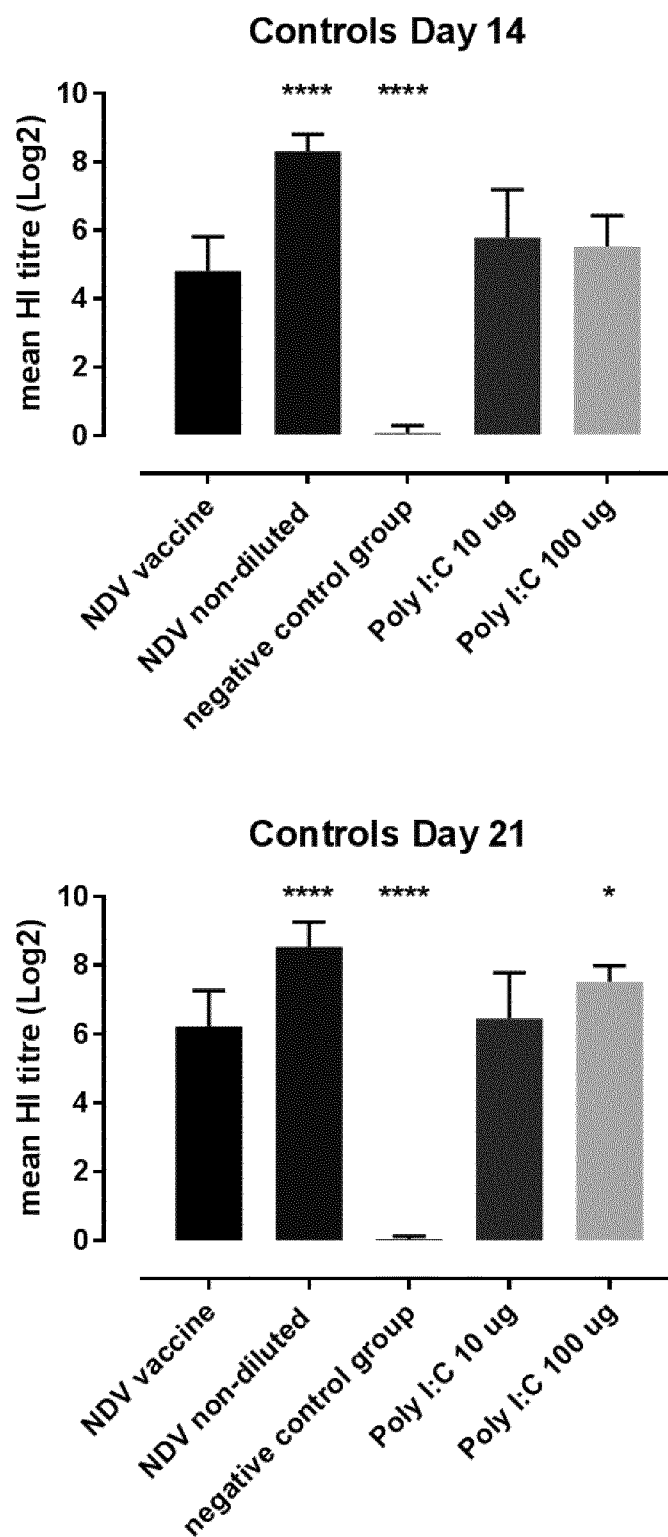
**FIG. 71**

**FIG. 72**

**FIG. 73**

**FIG. 74**

**FIG. 75**

**FIG. 76**

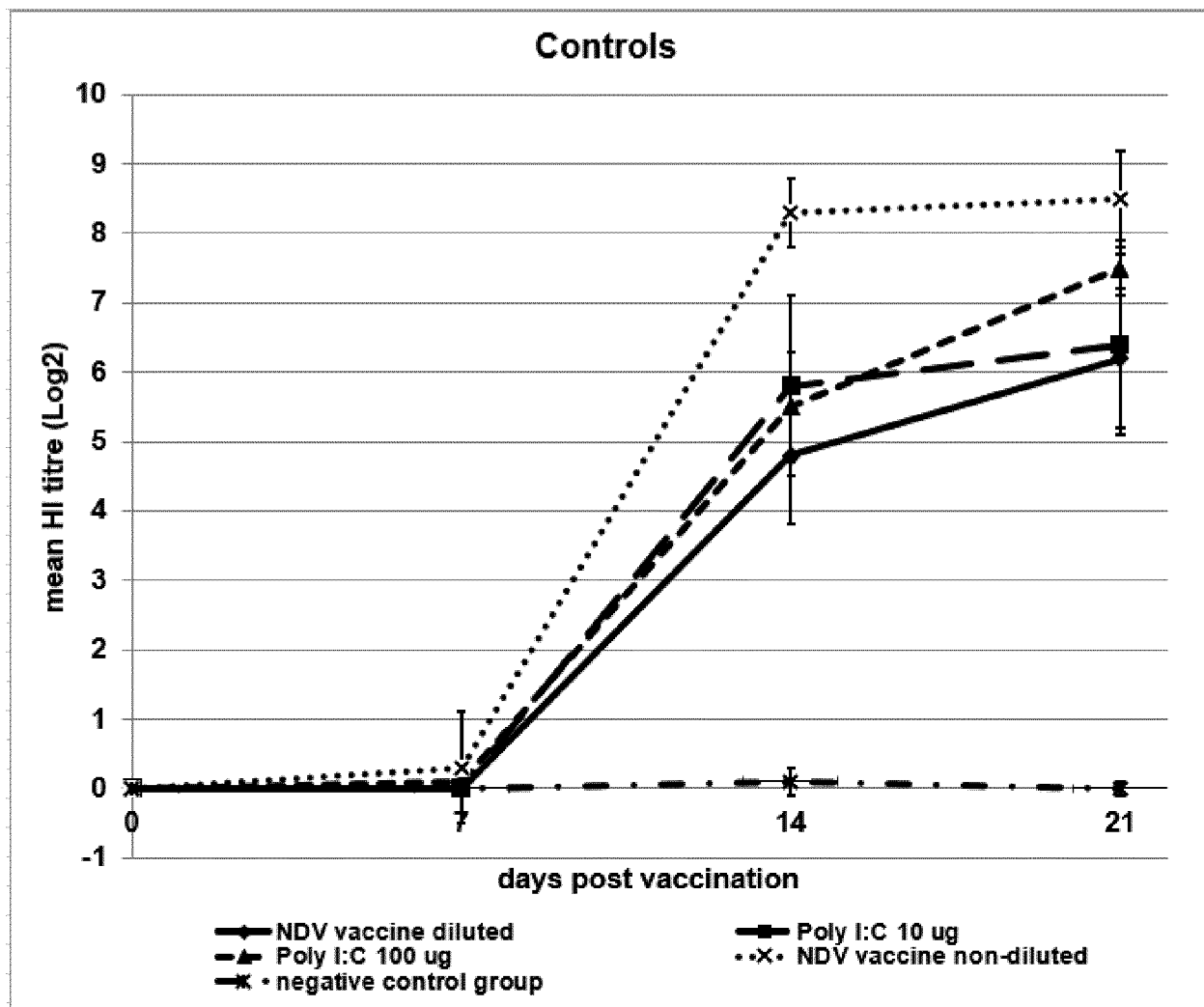
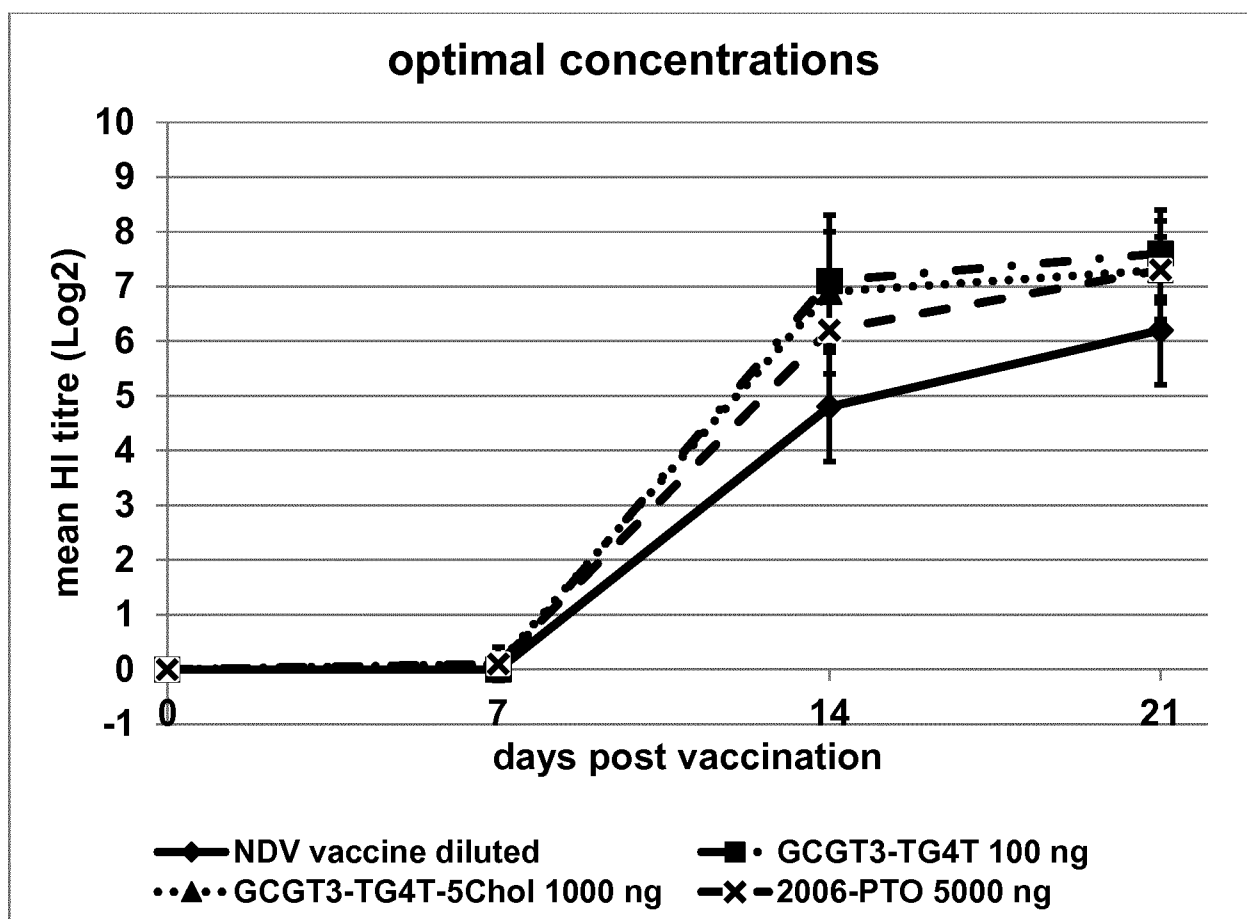
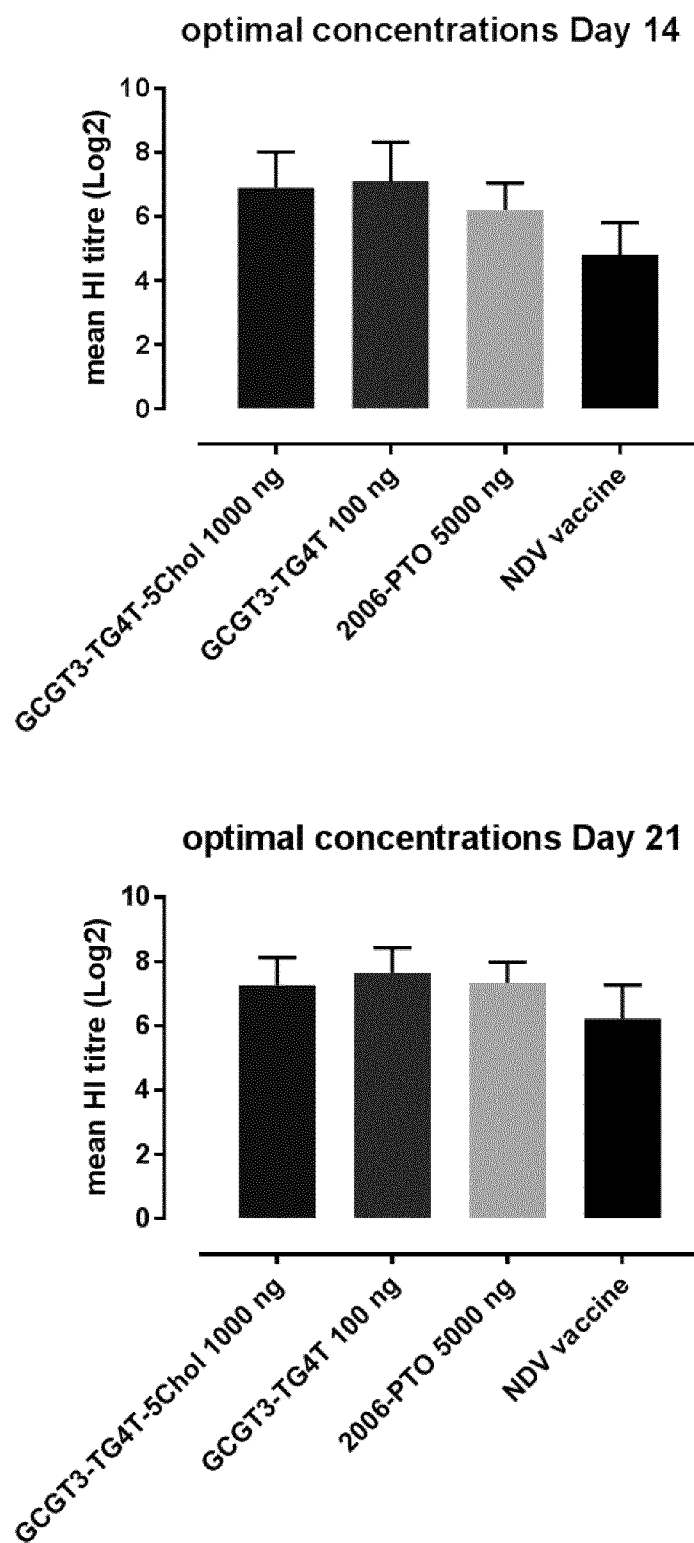


FIG. 77

**FIG. 78**

**FIG. 79**