Supporting information

Effects of G-quadruplex ligands on topology, stability, and immunostimulatory property of G-quadruplex based CpG Oligodeoxynucleotides

Anh Thi Tram Tu[a, b, c, d], Kazuaki Hoshi [a], Yue Ma[e], Taiji Oyama[f], Satoko Suzuki[f],

Kaori Tsukakoshi[e], Kazuo Nagasawa[e], Kazunori Ikebukuro[e], Tomohiko Yamazaki*[, a, b]

[a] Nanomedicine Group, Research Center for Functional Materials (RCFM), National Institute for Materials Science (NIMS), 1-2-1, Sengen, Tsukuba 305-0047, Japan

[b] Division of Life Science, Hokkaido University, Kita 10, Nishi 8, Kita-ku, Sapporo 060-0808, Japan

[c] Department of Magnetic and Biomedical Materials, Faculty of Materials Science, University of Science, Vietnam National University, 227 Nguyen Van Cu street, ward 4, district 5, Ho Chi Minh, 70000, Viet Nam

[d] Vietnam National University, Linh Trung ward, Thu Duc city, Ho Chi Minh City, 70000, Viet Nam

[e] Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16, Naka-cho, Koganei 184-8588, Japan

[f] JASCO Corporation, 2967-5, Ishikawamachi, Hachioji, Tokyo 192-8537, Japan

*Corresponding author: Tomohiko Yamazaki, Ph.D

Nanomedicine group, Research Center for Functional Materials (RCFM), National Institute for Materials Science (NIMS)

1-2-1 Sengen, Tsukuba, Ibaraki, 305-0047 JAPAN

Phone number: +81-29-859-2345

Fax: +81-29-859-2449

Email: YAMAZAKI.Tomohiko@nims.go.jp

I. Experimental section

Ultraviolet (UV) melting curves and thermal difference spectra (TDS)

A solution of 10 μM reconstructed GD3 was prepared in D-PBS. L2H2 and L2G2 stock solutions (10 mM) were diluted with sterile MiliQ water to a concentration of 1 mM. The reconstructed GD3 and G4 ligands were mixed to final concentrations of 2 μM and 8 μM, respectively. The G4 ligand/ODN molar ratio (R) was equal to 4 (R = 4). The mixtures were incubated at room temperature for 24 h before measurement.[1] UV melting curves and TDS measurements were obtained as previously described.[2] The baseline method was used to determine the melting temperature (T_m) of G4 CpG ODNs and the melting curves were measured at 295 nm.[3]

Polyacrylamide gel electrophoresis (PAGE)

Gel electrophoresis was conducted using 15% polyacrylamide gels 1 mm thick (ATTO, Tokyo, Japan) in 0.5× Tris-borate-EDTA (TBE) buffer (0.089M, pH 8.3-8.5, Takara Bio, Kusatsu, Japan) supplemented with 0 or 4 mM KCl, under a constant current of 21 mA at 4 °C. An ultralow molecular weight range DNA ladder (Invitrogen) was used as a marker. The bands in the gel were stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA). ssODN38mer (5'-GTCGTTTTGTCGTTTTGTCGTTTTGTCGTTTTGTCGTT-3') was used as a reference for linear CpG ODN comprising 38 nucleotides. ssODN38mer was diluted in 10 mM Tris-HCl at pH 7.4, then heated to 95 °C for 5 min and immediately put into ice to form a linear structure.

Sample preparation for size exclusion-high performance liquid chromatography (SE-HPLC)

GD3 was reconstructed at 20 μM in D-PBS. L2H2 and L2G2 stock solutions (10 mM) were diluted with sterile MiliQ water to a concentration of 1 mM. The reconstructed GD3 and G4 ligands were mixed to final concentrations of 5 μM and 20 μM, respectively (R = 4).

Stability in serum

Stability in serum was assayed to examine the nuclease resistance of GD3 alone and of GD3 in the presence of the G4 ligand. GD3 was reconstructed in D-PBS. L2H2 and L2G2 stock solutions (10 mM) were diluted with sterile MiliQ water to a concentration of 1 mM. The reconstructed GD3 and G4 ligands were mixed to final concentrations of 10 μM and 40 μM, respectively (R = 4), then the mixture was incubated at 25 °C for 24 h. Reconstructed G4 CpG ODNs (4 μL, 10 μM) alone or mixed with G4 ligands at R = 4 were added to 36 μL of D-PBS containing 20% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA) and incubated at 37 °C for 0, 1, 2, 4, and 24 h, then 4 μL of 250 mM ethylenediaminetetraacetic acid solution was added followed by heating to 80 °C for 2 min to stop the reaction. The processed ODNs were stored at 4 °C for PAGE analysis. The percentages of remaining ODNs relative to the amount of ODN at the beginning of the incubation were calculated based on the fluorescence intensities of the corresponding PAGE bands using Image Studio Lite software (LI-COR Biotechnology, Lincoln, NE, USA).

Cell cultures

The mouse macrophage-like RAW264 cell line (RCB0535) was purchased from RIKEN BioResource Center (Tsukuba, Japan) and maintained in minimum essential medium (MEM)

(Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich) and 1% (v/v) MEM non-essential amino acid (NEAA) solution (100×, Fujifilm Wako Pure Chemical Industries, Osaka, Japan) at 37 °C in a humidified incubator containing 5% CO_2.

Immunoprecipitation assay

GD3 was reconstructed in D-PBS. L2H2 and L2G2 stock solutions (10 mM) were diluted with sterile MiliQ water to a concentration of 1 mM. The reconstructed GD3 and G4 ligands were mixed to final concentrations of 10 μM and 40 μM, respectively (R = 4), then incubated at 25 °C for 24 h. A Dynabeads Protein G Immunoprecipitation kit (Invitrogen) and Recombinant Mouse TLR9Fc Chimera (mTLR9Fc, R&D Systems, Minneapolis, MN, USA) were used to examine the binding between mouse TLR9 to GD3 alone or to GD3 complexed with G4 ligands, as previously described.[2]

The eluted solutions were analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to detect mTLR9Fc binding on the dynabeads. The conditions were 15% polyacrylamide gel (ATTO), 1× Tris-glycine-SDS running buffer (TG-SDS, EMD Millipore Corp., Bedford, MA, USA) with Coomassie Brilliant Blue (CBB) (Nacalai Tesque, Kyoto, Japan) staining. The eluted solutions were subjected to PAGE to detect CpG ODNs binding to mTLR9Fc using a 10-20% polyacrylamide gel (ATTO) with 1× Tris-glycine running buffer. An ultralow molecular weight range DNA ladder (Invitrogen) was used as a marker. ODNs were visualized using SYBR Gold.

Cell viability assay

L2H2 and L2G2 stock solutions (10 mM) in DMSO were diluted with sterile MiliQ water to a concentration of 5 mM. Then, the G4 ligand solutions were diluted in D-PBS to a concentration

of 320 μM. RAW264 cells were seeded on a 96-well plate at a density of 1 × 10^5 cell/well (5.3 × 10^5 cell/mL, 190 mL) in culture medium containing 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated for 18 h before 10 μL of G4 ligand was added into the well. The final concentration of G4 ligand in the well is 16 μM, the final concentration of DMSO in the well is 0.16% v/v. The cells were incubated for 24 h at 37°C and 5% CO_2. Afterwards, the supernatant was removed completely, and the cells were washed with 200 μL PBS one time. Add 200 μL of CellTiter-Glo Reagent (Promega Corporation), which was diluted 2-fold in PBS, to the wells. The contents were mixed at room temperature for 12 minutes on an orbital shaker. Subsequently, the plate was allowed to incubate at room temperature for 10 minutes to stabilize luminescent signal. Next, we transfered 100 μL of reactant to half-area white microplate. Blank wells containing 50 μL of diluted CellTiter-Glo Reagent were prepared to obtain a value for background luminescence. Luminescence was recored using Ensight plate reader (PerkinElmer).

Stimulation of RAW264 cells

GD3 was reconstructed at 85.46 μM in D-PBS. L2H2 and L2G2 stock solutions (10 mM) were diluted with sterile MiliQ water to a concentration of 5 mM. The reconstructed GD3 and G4 ligands were mixed to final concentrations of 80 μM and 320 μM, respectively (R = 4), then incubated at 25 °C for 24 h. The compositions of the GD3/G4 ligand solutions are presented in **Table S1**. RAW264 cells on a 96-well plate were seeded at a density of 1 × 10^5 cell/well (5.3 × 10^5 cell/mL, 190 mL) in culture medium containing 100 units/mL penicillin, and 100 μg/mL streptomycin, then incubated for 18 h. The cells were then stimulated with 4 μM CpG ODNs by adding 10 μL of 80 μM ODNs alone or mixed with G4 ligands at R = 4. After 24 h-incubation, the relative transcript levels and secretion levels of the cytokines were determined.

Table S1 Composition of the GD3/G4 ligand solutions

Sample	V_{GD3} (µL)[a]	V_{L2H2} (µL)[b]	V_{L2G2} (µL)[b]	$V_{0.5\times DMSO}$ (µL)	$V_{MiliQ\ water}$ (µL)	Total volume (µL)
GD3	56.16	-	-	3.84	-	**60.00**
GD3/L2H2	56.16	3.84	-	-	-	**60.00**
GD3/L2G2	56.16	-	3.84	-	-	**60.00**

[a] Volume of GD3 solution at 85.46 µM.

[b] Volume of L2H2 or L2G2 solution at 5 mM.

Relative transcript levels of IL-6, and IL-12p40 in RAW264 cells, were examined by RT/RQ-PCR as described previously.[2] The sequences of the forward (FW) and reverse primer (RV) of murine cytokines are as follows:

GAPDH (FW: 5'-GTGGACCTCATGGCCTACAT-3'; RV: 5'-TGTGAGGGAGATGCTCAGTG-3'), IL-6 (FW: 5'-TCCTTCCTACCCCAATTTCC-3'; RV: 5'-CGCACTAGGTTTGCCGAGTA-3'), IL-12p40 (FW: 5'-GAAAGGCTGGGTATCGG-3'; RV: 5'-GGCTGTCCTCAAACTCAC-3'), IL-10 (FW: 5'-CGGGAAGACAATAACTGCACCC-3'; RV: 5'-CGGTTAGCAGTATGTTGTCCAGC-3'), and IFN-β (FW: 5'-GGTCCGAGCAGAGATCTTCA-3', RV: 5'-TCACTACCAGTCCCAGAGTCC-3').

The supernatants from RAW264 cells stimulated with GD3 or GD3 in complex with G4 ligands were collected by centrifuging (10,000 ×*g*) for 10 min at 4 °C. IL-6, IL-12p40, TNF-α, and IFN-β secretion levels in the supernatants were determined using a Mouse IL-6 ELISA

Ready-SET-Go kit (eBioscience, San Diego, CA, USA), OptEIA Set for mouse interleukin-12 (p40) (BD Biosciences Pharmingen, San Diego, CA, USA), TNF alpha Mouse Uncoated ELISA Kit (Thermo), and VeriKine Mouse IFN-β ELISA kit (VeriKin, Pestka Biomedical Laboratories), respectively, according to the manufacturer's instructions.

Statistical analysis

Statistical comparisons of the data were conducted by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for comparison with other groups, or by Dunnett's multiple comparisons test for comparison with a control group. All statistical analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, La Jolla, CA, USA). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, and p-value < 0.05 were considered statistically significant.

II. Figures and tables

Table S2 Melting temperatures of GD3 and GD3 in complex with G4 ligands [a]

ODNs	G4 Topology	Melting temperature (°C)[b]	References
GD3	Hybrid	41	[2]
GD3/L2H2	Hybrid	77	In this study
GD3/L2G2	Parallel	> 90	In this study

[a] GD3, GD3/L2H2 and GD3/L2G2 were prepared at a concentration of 2 μM in D-PBS with about 4 μM K^+ and 150 μM Na^+. Molar ratio $R = n_{G4ligand}:n_{GD3} = 4$.

[b] The melting temperatures of GD3, GD3/L2H2 and GD3/L2G2 were determined from the UV melting curves at 295 nm using the baseline method.

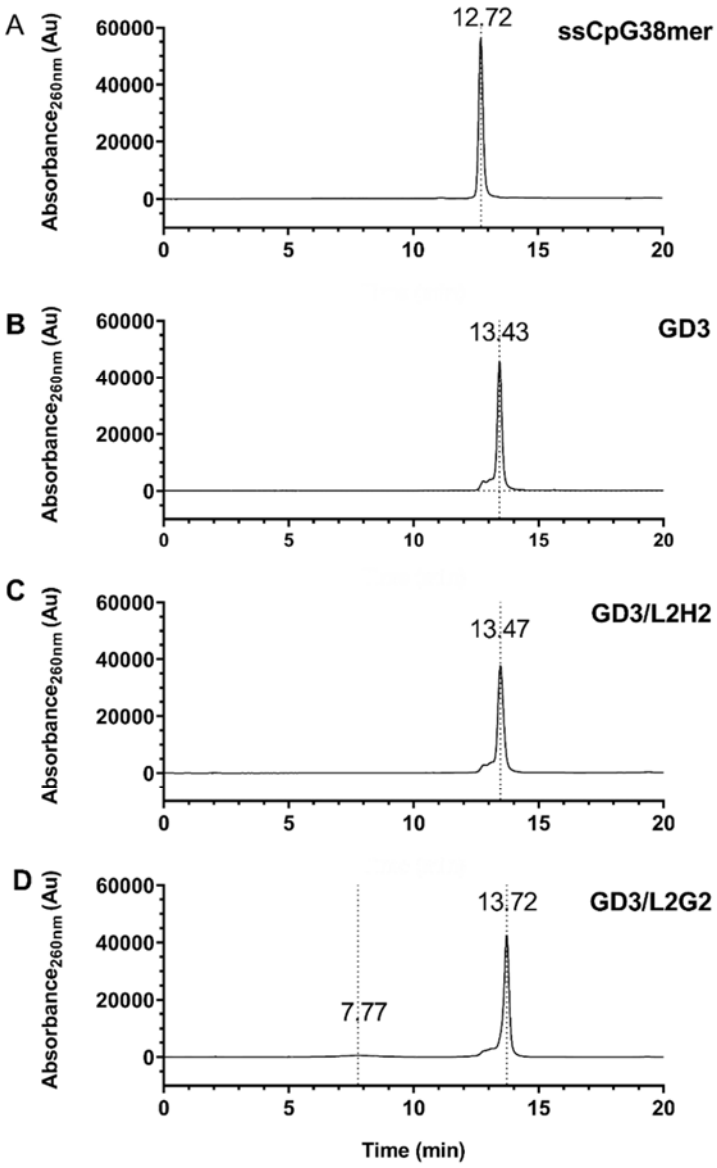

Figure S1 Size exclusion-HPLC chromatograms of (A) ssCpG38mer, (B) GD3, (C) GD3/L2H2, (D) GD3/L2G2.

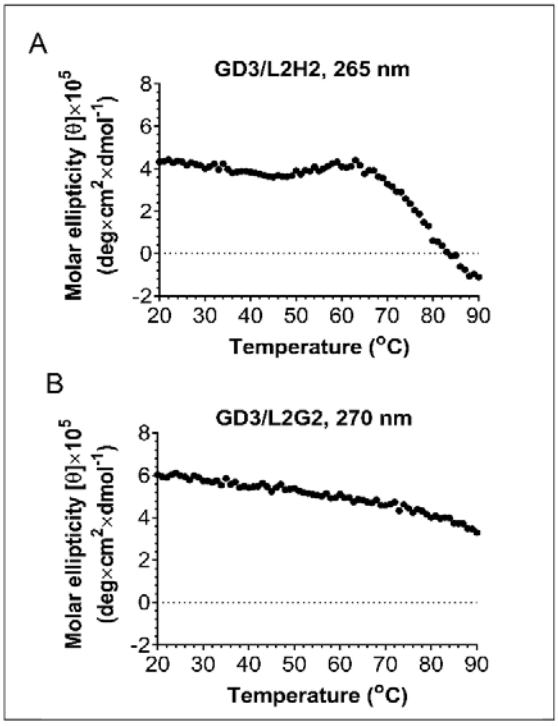

Figure S2 Circular dichroism (CD) melting curves of GD3 in complex with G4 ligands at R = $n_{G4\text{-}ligand}$:n_{GD3} = 4. (A) GD3/L2H2 and (B) GD3/L2G2. The molar ellipticity values were monitored during heating from 20°C to 90°C at 265 nm, and 270 nm for GD3/L2H2, and GD3/L2G2, respectively.

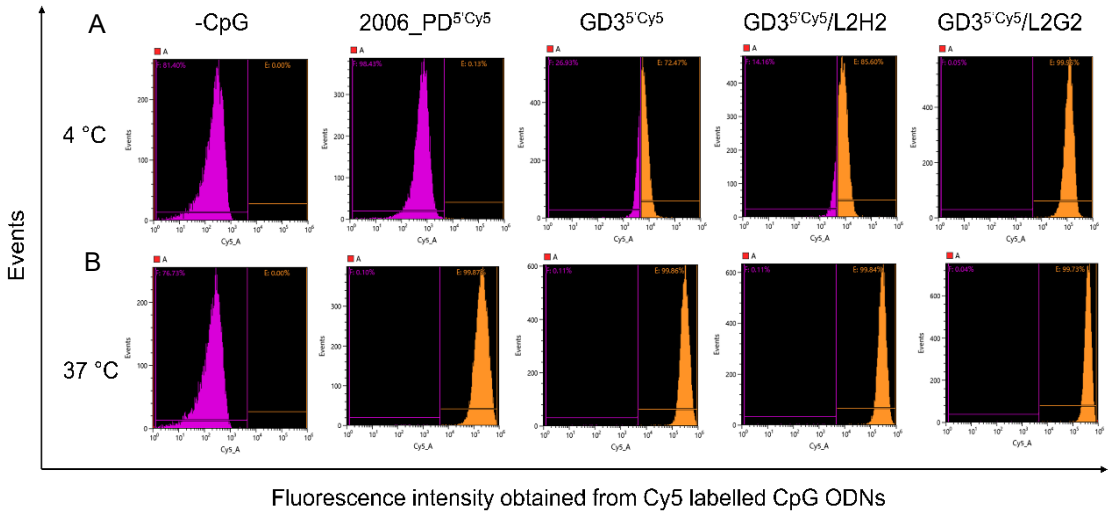

Figure S3 Histogram analysis of the fluorescence intensity of RAW264 cells treated with CpG ODN/G4 ligand at (A) 4 °C and (B) 37 °C.

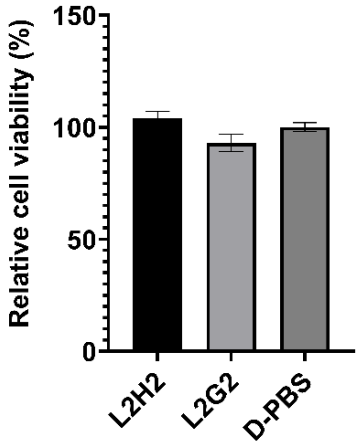

Figure S4 Viability of RAW264 cells incubated with G4 ligands for 24 hours. Final concentration of G4 ligands in culture medium is 16 μM, under the same condition as in the cytokine induction experiment. Data represent mean ± SD (n = 5).

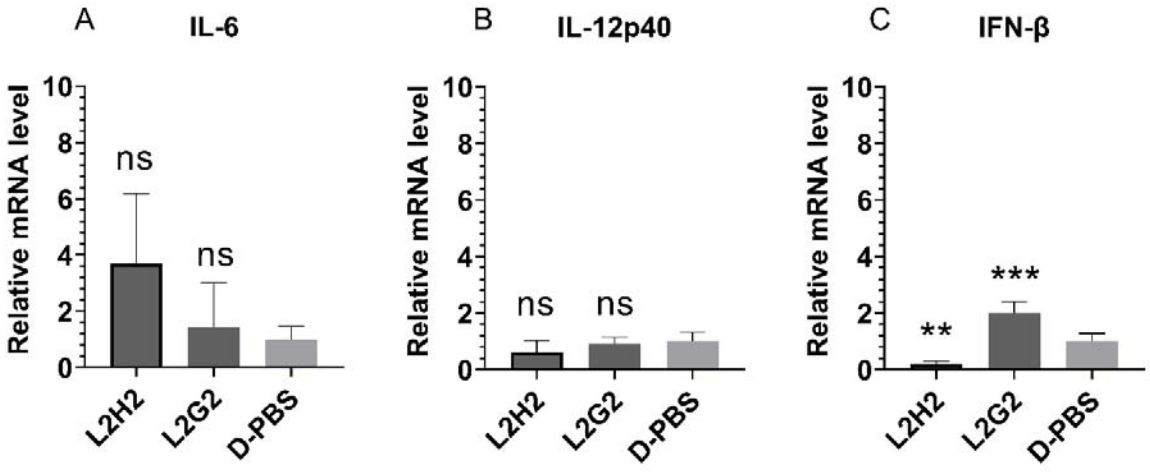

Figure S5 G4 ligands did not induce immune responses. Relative mRNA levels of (A) *IL-6*, (B) *IL-12*, and (C) *IFN-β* in mouse macrophage-like RAW264 cells after 24 h of stimulation with 16 μM of L2H2-6OTD or L2G2-2M2EG-6OTD. The relative mRNA level compared to [D-PBS] was calculated. Data represent mean ± SD (n = 5). ***$p < 0.001$, **$p < 0.01$, ns (not significantly different) p>0.05 (one-way ANOVA, Dunnett's multiple comparisons test for comparison with the D-PBS control group).

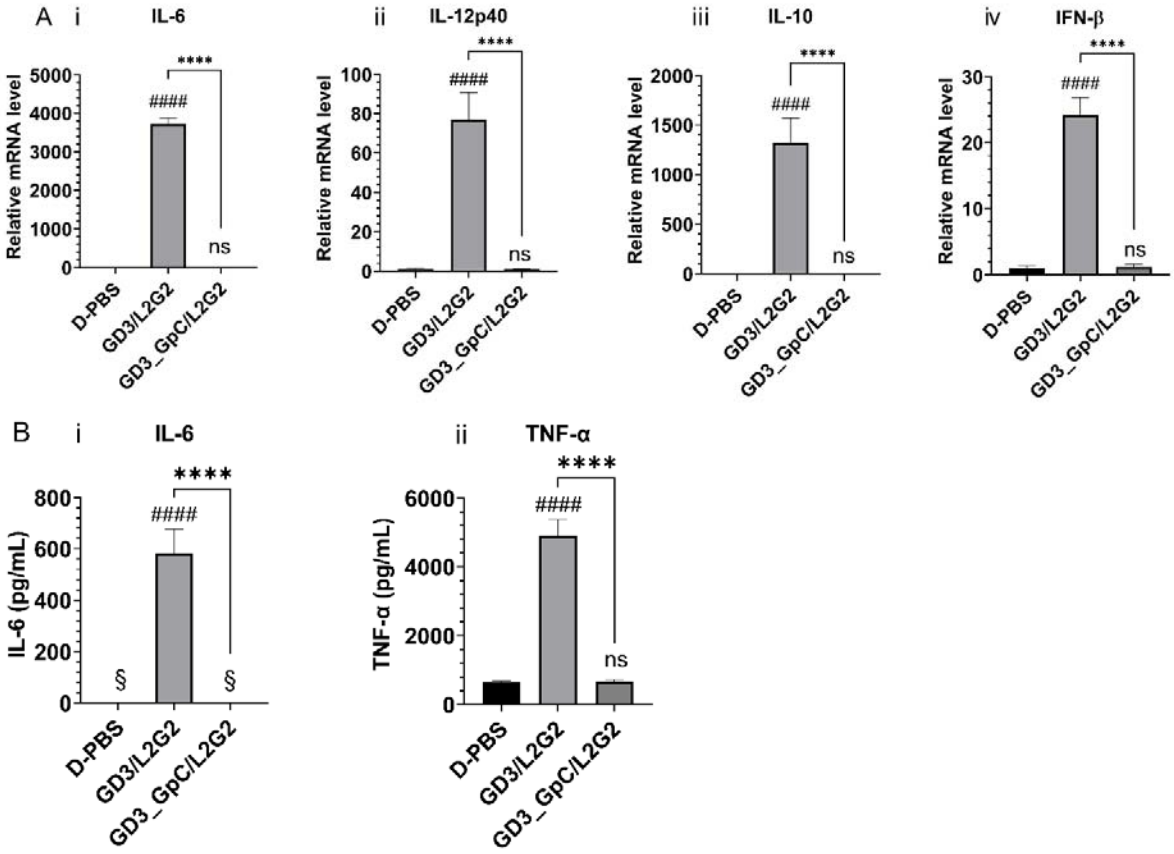

Figure S6 Dependence on CpG motifs of cytokine induction of GD3/L2G2. (A) Relative mRNA levels of (i) *IL-6*, (ii) *IL-12p40*, (iii) *IL-10*, and (iv) *IFN-β* expressed in the cells; (B) The protein level of (i) IL-6, and (ii) TNF-α secreted into the culture medium. Mouse macrophage-like RAW264 cells were stimulated with 4 μM GD3 or GD3_GpC in complex with L2G2 at R = $n_{G4\text{-}ligand}$:n_{GD3} = 4. The relative mRNA level compared to [D-PBS] was calculated. Data represent mean ± SD (n = 5). ****p < 0.0001, ns (not significantly different) p >0.05 (*, one-way ANOVA, Tukey's multiple comparisons test for comparison with other groups; #, Dunnett's multiple comparisons test for comparison with the D-PBS control group). §: lower detection limit (3.9 pg/mL).

Sequence of ODNs (The backbone consists entirely of phosphorodiesters.)

GD3 : 5’-GGGTTGGGGTCGTTTTGTCGTTTTGTCGTTGGGTTGGG-3’.

GD3_GpC: 5’-GGGTTGGGGTGCTTTTGTGCTTTTGTGCTTGGGTTGGG-3’.

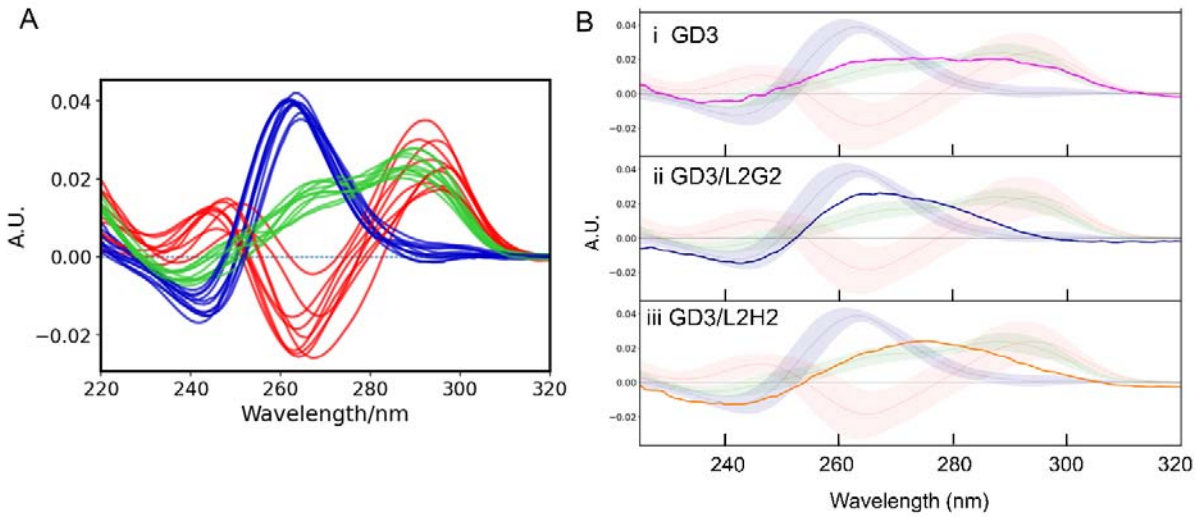

Figure S7 (A) CD spectra of the library of 30 reference G-quadruplex ODNs. Blue, green and red spectra indicate parallel, hybrid and anti-parallel structures, respectively. (B) Comparison of CD spectra of (i) GD3, (ii) GD3/L2G2, and (iii) GD3/L2H2 with parallel, antiparallel, and hybrid reference spectra. The spectrum drawn in a lighter color in the background and the colored area around the spectrum indicate the average spectrum of each structure and its 2σ limit. Blue, green, and red indicate parallel, hybrid, and anti-parallel, respectively.

References

(1) Ma, Y.; Tsushima, Y.; Sakuma, M.; Sasaki, S.; Iida, K.; Okabe, S.; Seimiya, H.; Hirokawa, T.; Nagasawa, K. Development of G-Quadruplex Ligands for Selective Induction of a Parallel-Type Topology. *Org. Biomol. Chem.* **2018**, *16* (40), 7375–7382. https://doi.org/10.1039/C8OB01702F.

(2) Tu T. T., A.; Hoshi, K.; Ikebukuro, K.; Hanagata, N.; Yamazaki, T. Monomeric G -Quadruplex-Based CpG Oligodeoxynucleotides as Potent Toll-Like Receptor 9 Agonists. *Biomacromolecules* **2020**, *21* (9), 3644–3657. https://doi.org/10.1021/acs.biomac.0c00679.

(3) Mergny, J. L.; Lacroix, L. UV Melting of G-Quadruplexes. *Curr. Protoc. Nucleic Acid Chem.* **2009**, No. SUPPL. 37, 1–15. https://doi.org/10.1002/0471142700.nc1701s37.