

Elucidation and optimization of the interaction of
nanostructured DNA and immune cells

-Digest-

2017

Shozo OHTSUKI

Contents

Preface	1
Chapter I	2
I Introduction	3
Chapter I	
Section 1	4
I-1-1 Introduction	4
I-1-2 Materials and Methods	4
I-1-3 Results	9
I-1-3-a. Construction of DNA nanostructures	9
I-1-3-b. T _m and apparent size of DNA nanostructures	11
I-1-3-c. CD spectra of DNA nanostructures	12
I-1-3-d. Stability of DNA nanostructures in serum	12
I-1-3-e. Uptake of Alexa Fluor 488-labeled DNA nanostructures in RAW264.7 cells	13
I-1-3-f. TNF- α release from RAW264.7 cells after addition of CpG DNA nanostructures	13
I-1-3-g. DNA uptake and IFN- α release after addition of DNA nanostructures to human PBMCs	14
I-1-4 Discussion	14
Chapter I	
Section 2	18
I-2 Summary	18
Chapter I	
Section 3	20
I-3 Summary	20
Chapter II	22
II Introduction	23
Chapter II	
Section 1	24
II-1 Summary	24
Chapter II	
Section 2	25
II-2 Summary	25
Chapter III	26
III-1 Introduction	27
III-2 Materials and Methods	28

III-3 Results	31
III-3-a. Establishment of HEK-Blue hTLR9/hMSR-1 cells	31
III-3-b. Evaluation of the functions of HEK-Blue hTLR9/hMSR-1 cells	32
III-3-c. Experiments using HEK-Blue hTLR7 cells	34
III-4 Discussion	34
Summary.....	37
Acknowledgements	38
List of Publications.....	40
Other Publications	41
References	42

Preface

Nucleic acids are the key molecules of signal transduction in humans and other organisms. Genetic information is stored in the genomic DNA, and vital activity is constantly carried out through RNA transcribed as necessary. In recent years, research on nucleic acid drugs has progressed rapidly. Various classes of nucleic acid drugs are currently used or under development^{1,2}, and some nucleic acid-based drugs have been approved for clinical use³. The negative charge of nucleotide backbone greatly limits the cellular uptake of nucleic acid-based drugs. To overcome this limitation, many chemical modifications on these nucleic acid-based drugs are applied to increase their nuclease resistance and cellular uptake by target cells⁴⁻⁶.

Unmethylated cytosine-phosphate-guanine dinucleotide with appropriate flanking sequences, or CpG motif, is a pathogen-associated molecular pattern and a ligand for Toll-like receptor 9 (TLR9) expressed in various mammalian immune cells⁷⁻⁹. The binding of CpG DNA, a DNA containing the CpG motif, to the receptor leads to the production of proinflammatory cytokines, including tumor necrosis factor (TNF)- α , interleukin (IL)-6, and interferon (IFN)- α ^{10,11}. Therefore, CpG DNA has attracted attention in the treatment of cancer, virus infection, and allergic diseases¹²⁻¹⁶. However, CpG DNA has not been clinically applied yet.

DNA has a unique property to hybridize with its complementary sequence. More recently, DNA nanotechnology has greatly increased the potential of DNA as a building block for DNA assemblies. So far, some unique structures have been developed using DNA¹⁷⁻²¹. These DNA structures could be used as therapeutic agents or drug delivery systems. My laboratory reported that multi-branched, polypod-like structured DNA, or polypodna, prepared using three or more oligodeoxynucleotides (ODNs), is a useful DNA nanostructure for the delivery of natural phosphodiester CpG DNA to mouse macrophage-like RAW264.7 cells, mouse dendritic DC2.4 cells, mouse bone marrow-derived dendritic cells, and human peripheral blood mononuclear cells²²⁻²⁵. The polypod-like structure was found to be quite effective for the delivery of DNA to these immune cells.

However, there is little information about the interaction of nanostructured DNA and target cells. Therefore, I aimed to develop efficient delivery systems to immune cells using nanostructured DNA. To achieve this, the interaction of nanostructured DNA and immune cells needs to be elucidated and optimized. In Chapter I, I elucidated the structural properties of nanostructured DNA that are required for the interaction with immune cells. In Chapter II, I focused on the effect of the structural rigidity or structural compactness of nanostructured DNA on the interaction with immune cells using DNA origami or DNA supramolecules. Finally, in Chapter III, I elucidated cell surface receptors that are involved in the cellular uptake of nanostructured DNA.

In this thesis, the results are presented in the following three chapters.

Chapter I

Elucidation of the structural properties of nanostructured DNA required for the interaction with immune cells

Recent advances in DNA nanotechnology have greatly increased the potential of DNA strands as a building block for DNA assemblies²⁶. A variety of uniquely structured DNA assemblies, such as DNA origami-based nanostructures¹⁷, DNA polyhedral¹⁸, and DNA hydrogel^{20,27}, have been reported. Some of these have been examined as pharmaceuticals or drug delivery systems^{26,28}.

My laboratory previously demonstrated that nanostructure formation increased CpG DNA-induced immune activation^{22-25,29}. However, detailed studies on the interaction of nanostructured DNA with cells are needed to develop nanostructured DNA-based delivery systems for efficient delivery of nucleic acid drugs including CpG DNA to target cells.

In this chapter, I aimed to elucidate the structure-activity relationship of self-assembled nanostructured DNA as delivery vehicles for target cells. I designed three different nanostructured DNAs using four 55-mer oligodeoxynucleotides (ODNs), that is tetrapod-like structured DNA (tetrapodna), tetrahedral DNA (tetrahedron), and tetragonal DNA (tetragon), and compared their potencies. These three nanostructured DNAs were compared in terms of preparation efficiency, physicochemical property, and interaction with the cells. Additionally, I hypothesized bend type DNA could be efficiently taken up by immune cells. Moreover, for clinical use, it is necessary to evaluate the cellular uptake of nanostructured DNAs by cells other than target cells. Therefore, I selected tripodna, terapodna, hexapodna, terahedron, and tetragon to examine their uptake in several types of cells.

In this chapter, the results are presented in the following three sections.

Chapter I

Section 1

Optimal arrangement of four short DNA strands for delivery of immunostimulatory nucleic acids to immune cells

I-1-1 Introduction

Apart from the use of chemically stabilized DNA/RNA analogues, such as phosphorothioate DNA^{30,31}, several attempts have been made to increase the activity of natural phosphodiester CpG DNA through efficient delivery of the DNA to the target immune cells. Separately, Li et al. reported that DNA tetrahedron, designed using four ODNs, was useful for the delivery of CpG DNA to RAW264.7 cells³². These previous studies have clearly demonstrated that nanosized DNA assemblies are suitable systems for the delivery of CpG DNA to immune cells for immunostimulation. However, no systematic studies have been carried out to elucidate the optimal structure of such DNA assemblies for the delivery of immunostimulatory CpG DNA to immune cells.

In this section, I examined the importance of the structure of nanosized DNA assemblies in the preparation efficiency as well as the stimulation of immune cells. Since previous study from my laboratory demonstrated that the number and length of ODNs were key parameters for the physicochemical and biological properties of polypodnas²⁴, these parameters were fixed in order to extract the structure-dependent differences among the nanosized DNA assemblies. To this end, tetrahedral DNA (tetrahedron) consisting of four 55-mer ODNs, reported by Li et al.³², was selected as one of the DNA assemblies for comparison. To minimize the difference in the sequences, one of the ODNs for the tetrahedron (the common ODN) was used to newly design tetrapodna consisting of four 55-mer ODNs. In addition, a tetragonal DNA (tetragon) was also designed with the common ODN and three other 55-mer ODNs. These three DNA nanostructures were compared in terms of preparation efficiency, physicochemical property, cellular uptake, and immunostimulatory activity. RAW264.7 cells and human peripheral blood mononuclear cells (PBMCs) were used for cellular interaction of these DNA nanostructures.

I-1-2 Materials and Methods

Chemicals

Roswell Park Memorial Institute (RPMI) 1640 medium was obtained from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). Opti-MEM was purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories, Inc. (South Logan, UT, USA). The 100 base pair (bp) DNA ladder was purchased from Takara Bio (Otsu, Japan). All other chemicals were the highest grade available and used without further purification.

Cell culture

The murine macrophage-like cell line RAW264.7 was cultured in RPMI medium supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine at 37°C in humidified air containing 5% CO₂. RAW264.7 cells were then plated on 24-well or 96-well culture plates at a density of 5×10^5 cells/mL and cultured for 24 h prior to use.

Isolation of human peripheral blood mononuclear cells

All experiments using human primary cells were approved by the institutional review board at the Graduate School of Medicine, Kyoto University, and following the tenets of the Declaration of Helsinki. Human PBMCs were obtained from healthy donors after obtaining their written informed consent. PBMCs were isolated from peripheral blood by density gradient centrifugation using Lympholyte-H Cell Separation Media (Cedarlane Laboratories Ltd., Netherlands) as previously reported³³. Cells collected were replated on 48-well culture plates at a density of 6×10^5 cells/well in RPMI medium.

Oligodeoxynucleotides

All ODNs used were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The sequences of the ODNs used are listed in Table 1. ODN-1-1-1 was the common ODN used for all three preparations. Each ODN, such as tetrapodna-1-1-2, tetrahedron-1-1-2, and so on, was identified with the name of the DNA assembly and the ODN number. For cellular uptake experiments, ODN-1-1-1 labeled with Alexa Fluor-488 at the 5' end (Alexa ODN-1-1-1) was purchased from Japan BioService Co., Ltd. (Saitama, Japan). CpG ODN-1 was designed in such a way that a 25-base ODN containing a potent CpG motif was linked to the 5'-end of the ODN-1-1-1, and used for cytokine release experiments. AFM-tetrapodna-1-1-1, AFM-tetrapodna-1-1-3, AFM-tetrahedron-1-1-2, AFM-tetrahedron-1-1-3, AFM-tetragon-1-1-2, and AFM-tetragon-1-1-3 were used for AFM imaging.

Preparation of DNA nanostructures

ODNs dissolved in an annealing buffer (TE buffer; 10 mM Tris-HCl, pH 8, 1 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM magnesium chloride) were mixed at a final concentration of 0.5–20 µM for each ODN. The annealing buffer was used to prepare all the DNA samples. The mixtures were then incubated at 95°C for 2 min and slowly cooled down to 4°C using a thermal cycler. The annealing condition for tetrahedron was optimized in preliminary experiments, and a different condition from the original one³² was used to minimize the formation of aggregates at high DNA concentrations. Each preparation was analyzed at 37°C by the MCE-202 MultiNA microchip electrophoresis system for DNA/RNA analysis (Shimadzu, Kyoto, Japan) or by 6% polyacrylamide gel electrophoresis. Double-stranded (ds) DNA was prepared by annealing of ODN-1-1-1 and ODN-1-1-1'.

Atomic force microscopy imaging of DNA nanostructures

Atomic force microscopy (AFM) images were obtained on a high-speed AFM system (Nano Live Vision, RIBM, Tsukuba, Japan) using a silicon nitride cantilever (BL-AC10EGS; Olympus, Tokyo, Japan) as reported previously³⁴. All the DNA nanostructures were immobilized to the DNA frame using the two long single-stranded sequences on the 5'-end of the two ODNs of each DNA nanostructure. In brief, a DNA sample was adsorbed on a freshly cleaved mica plate pretreated with 0.1% aqueous 3-aminopropyltriethoxysilane for 5 min at room temperature and then washed three times with a buffer solution containing 20 mM Tris and 10 mM MgCl₂. Scanning was performed in the same buffer solution.

Measurement of melting temperature

The melting temperatures (T_m) were obtained by measuring the absorbance of tetrapodna, tetrahedron, and tetragon in TE buffer containing 50 mM MgCl₂ at 260 nm with a Shimadzu UV-1600 PC spectrometer (Shimadzu, Kyoto, Japan) equipped with a TMSPC-8 temperature controller²⁹.

Dynamic light scattering analysis

The apparent sizes of tetrapodna, tetrahedron, and tetragon were determined by the dynamic light scattering method using a Malvern Zetasizer 3000HS (Malvern Instruments, Malvern, UK) at 20°C. The measurement was repeated more than eight times, and the results were expressed as the mean ± SD of the eight measurements.

Circular dichroism spectra of DNA nanostructures

The circular dichroism (CD) spectra of DNA were recorded using a JASCO-820 type spectropolarimeter (JASCO, Tokyo, Japan) with a 0.1 cm path-length quartz cell at 20°C. The DNA samples after annealing were diluted with 50 mM MgCl₂-containing TE buffer to a final DNA concentration of 68 µg/mL. The CD spectra of DNA were measured in the range of 200 to 320 nm.

Stability of DNA nanostructures in 50% FBS

DNA nanostructures (68 µg/mL) were mixed with non-heat-inactivated FBS of equal volume and incubated at 37°C. After 0, 2, 4, 8, 12, or 24 h of incubation, a 10 µL aliquot of the sample solution was transferred to plastic tubes and mixed with 20 µL of 0.5 M EDTA solution to stop the degradation, and then stored at -20°C until use. These samples were run on 9% polyacrylamide gel electrophoresis (PAGE) at 4°C and stained with SYBR Gold (Life Technologies). The density of the DNA bands was quantitatively evaluated using the Multi Gauge software (Fujifilm, Tokyo, Japan).

Cellular uptake of DNA

RAW264.7 cells or PBMCs on 96 or 48-well plates at a density of 5×10^4 or 6×10^5 cells/well were incubated with Alexa Fluor 488-labeled single-stranded DNA (ssDNA), tetrapodna, tetrahedron, or tetragon diluted in 0.1 mL of Opti-MEM for 2 or 4 h at 37°C. Cells were then washed three times with 200 or 400 μ L of phosphate-buffered saline and harvested. Then, the fluorescent intensity of cells was determined by flow cytometry (FACS Calibur or Gallios Flow Cytometer, BD Biosciences, NJ, USA) using CellQuest software (version 3.1, BD Biosciences) or Kaluza software (version 1.0, BD Biosciences), and the mean fluorescence intensity (MFI) was calculated.

TNF- α release from RAW264.7 cells

RAW264.7 cells were seeded into 96-well plates at a density of 5×10^4 cells/well. Then, CpG ssDNA, CpG tetrapodna, CpG tetrahedron, or CpG tetragon was diluted in 0.1 mL of Opti-MEM and added to the cells. The cells were incubated at 37°C for 8 h, and the supernatants were retrieved and stored at -80°C until use. The levels of TNF- α in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using OptEIA™ sets (BD Biosciences).

IFN- α release from human PBMCs

PBMCs were seeded into 48-well plates at a density of 6×10^5 cells/well. Then, CpG ssDNA, CpG tetrapodna, CpG tetrahedron, or CpG tetragon was diluted in 0.2 mL of Opti-MEM and added to the cells. The cells were incubated at 37°C for 24 h, and the supernatants were retrieved and stored at -80°C until use. The levels of human IFN- α were determined using the IFN- α -ELISA module set (Bender Med Systems, Vienna, Austria). Chloroquine (Sigma-Aldrich, St. Louis, MO, USA) was used as a control for TLR involvement PBMCs. Cells were incubated with or without 100 μ M chloroquine.

Statistical analysis

Differences were statistically evaluated by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons. A P value of <0.05 was considered statistically significant.

Table 1. The sequences of ODNs for DNA nanostructures.

Name	Sequences (5' → 3')	Length (mer)
ODN-1-1-1	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGC CGCCATAGTA	55
Alexa ODN-1-1-1	*ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAG CCGCCATAGTA	55
ODN-1-1-1'	TACTATGGCGGCTCTTCTCGTGTAGCAAGCTGTAATGTTTCAGAC TTAGGAATGT	55
tetrapodna-1-1-2	TTACTATGGCGGCTCTTCTCGTGTAGCATAGTGTCTTTTATCAC CAGGCAGTTG	55
tetrapodna-1-1-3	TCAACTGCCTGGTGATAAAAACGACACTACGTGGGAATCTTGACAG GTCATCAGCC	55
tetrapodna-1-1-4	TGGCTGATGACCTGTCAAGATTCCCACGAGCTGTAATGTTTCAGA CTTAGGAATG	55
tetrahedron-1-1-2	TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAG GGTCCAATAC	55
tetrahedron-1-1-3	TCAACTGCCTGGTGATAAAAACGACACTACGTGGGAATCTACTATG GCGGCTCTTC	55
tetrahedron-1-1-4	TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCTTTGTATTGG ACCCTCGCAT	55
tetragon-1-1-2	CTGTAATGTTTCAGACTTAGGAATGTTTTGAAGTGACGCCAGAGT AGATTCCCAC	55
tetragon-1-1-3	GTGTCGTTTTTATCACCAGGCAGTTGATTTTACTATGGCGGCTCTT CTCGTGTAGC	55
tetragon-1-1-4	TCAACTGCCTGGTGATAAAAACGACACTTTGTGGGAATCTACTCTG GCGTCACTTC	55
CpG ODN-1-1-1	TCCAT GACGTT CCTGACGTTTTTTTACATTCCCTAAGTCTGAAACA TTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA	80
AFM-tetrapodna-1-1-1	ATAAGAATAAACACCGCATCATTTATAACATTCCCTAAGTCTGAAAC ATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA	81
AFM-tetrapodna-1-1-3	CGAGCTGAAAAGGTGGCATCATTTATATCAACTGCCTGGTGATAAA ACGACACTACGTGGGAATCTTGACAGGTCATCAGCC	81

AFM-tetrahedron-1	ATAAGAATAAACACCCGCATCATTATACATCATCATCATGCTCAAT	99
-1-2	ATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGG GTCCAATAC	
AFM-tetrahedron-1	CGAGCTGAAAAGGTGGCATCATTATACATCATTGTATGACTATAT	99
-1-3	CAACTGCCTGGTGATAAAAACGACACTACGTGGGAATCTACTATGG CGGCTCTTC	
AFM-tetragon-1-1-	ATAAGAATAAACACCCGCATCATTATACATCATCATCATGCTCTGT	96
2	AATGTTTCAGACTTAGGAATGTTTTGAAGTGACGCCAGAGTAGAT TCCCAC	
AFM-tetragon-1-1-	CGAGCTGAAAAGGTGGCATCATTATACATCATTGTATGACTGTGT	96
3	CGTTTTATCACCAGGCAGTTGATTTTACTATGGCGGCTCTTCTCG TGTAGC	

All ODNs have a phosphodiester backbone. The asterisk (*) indicates the position of Alexa Fluor-488 modification. Underlined is the CpG motif (GACGTT).

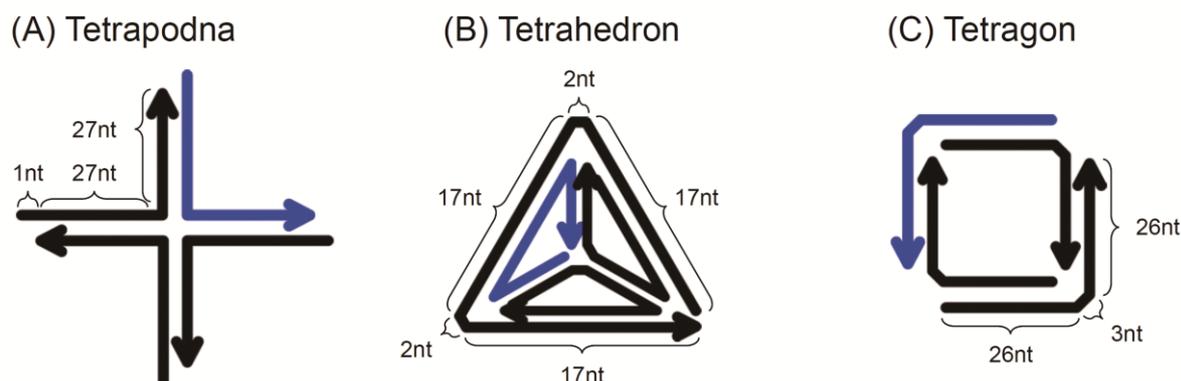


Figure 1. Schematic presentation of tetrapodna, tetrahedron, and tetragon. The blue-colored arrows are the common ODN (ODN1-1-1). The arrowheads represent the 3'-end of each ODN.

I-1-3 Results

I-1-3-a. Construction of DNA nanostructures

Figure 1 shows the schematic images of tetrapodna, tetrahedron, and tetragon. The blue-colored lines indicate the common ODN (ODN-1-1-1) for all preparations. Figure 2A shows the MultiNA analysis of tetrapodna, tetrahedron, and tetragon prepared at various DNA concentrations (0.5–20 μ M). A fixed amount of DNA (68 ng) was run on electrophoresis. Tetrapodna had a major single band of around 140 bp at all the DNA concentrations examined (Figure 2A, lanes 1–5), indicating that tetrapodna was successfully formed with high efficiency by simple annealing of ODNs. On the other hand, the band for tetrahedron, which was detected around 90 bp, became weaker with increasing DNA concentrations at preparation (Figure 2A, lanes 6–10). There were several bands for tetragon prepared at high DNA concentrations (Figure 2A, lanes 11–15).

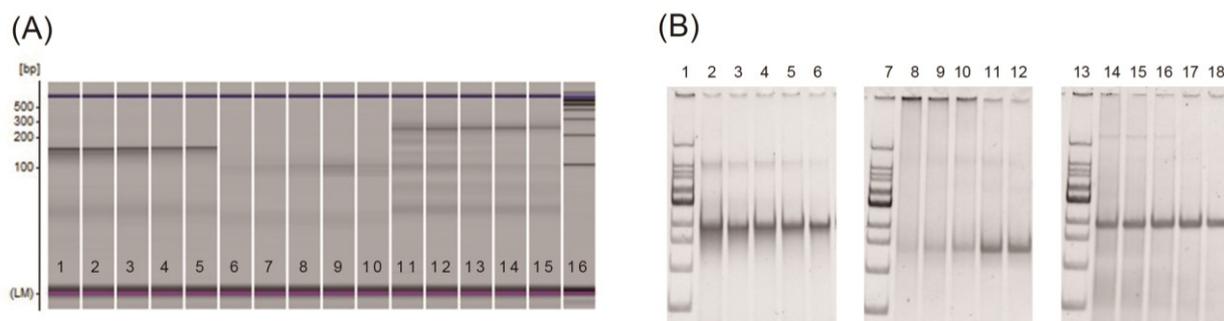


Figure 2. Electrophoretic analysis of tetrapodna, tetrahedron, and tetragon. (A) Aliquots of tetrapodna, tetrahedron, and tetragon (68 ng) prepared at 0.5–20 μM DNA were run on a MultiNA microchip electrophoresis system. Lanes 1–5, tetrapodna (20, 10, 5, 1, and 0.5 μM); lanes 6–10, tetrahedron (20, 10, 5, 1, and 0.5 μM); lanes 11–15, tetragon (20, 10, 5, 1, and 0.5 μM); lane 16, 100-bp DNA ladder. (B) Aliquots of tetrapodna, tetrahedron, and tetragon (68 ng) prepared at 0.5–20 μM DNA were run on a PAGE. Lanes 1, 7, and 13, 100-bp ladder, lanes 2–6, tetrapodna (20, 10, 5, 1, and 0.5 μM); lanes 8–12, tetrahedron (20, 10, 5, 1, and 0.5 μM); lanes 14–18, tetragon (20, 10, 5, 1, and 0.5 μM).

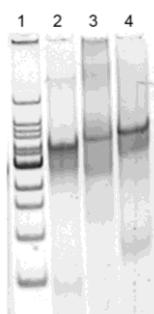


Figure 3. Electrophoretic analysis of tetrapodna, tetrahedron, and tetragon for AFM. Aliquots of tetrapodna, tetrahedron, and tetragon for AFM prepared at 1 μM DNA were run on a PAGE. Lane 1, 100-bp ladder; lane 2, tetrapodna; lane 3, tetrahedron; lane 4, tetragon.

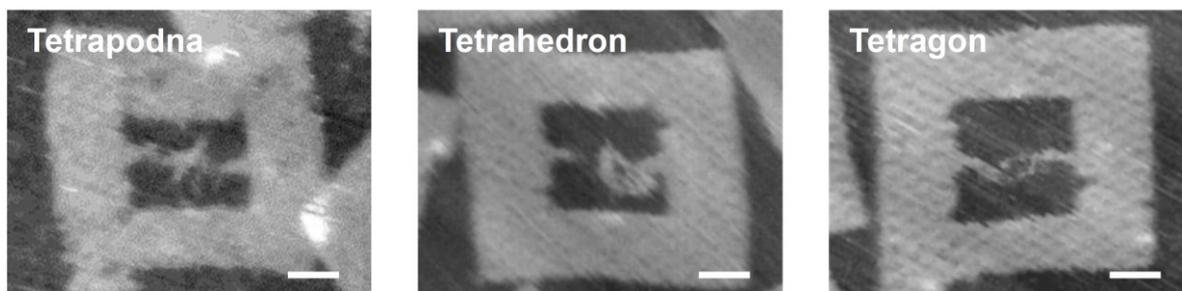


Figure 4. AFM images of tetrapodna, tetrahedron, and tetragon. The white rectangles with a window represent DNA origami frames. All DNA samples were annealed at a DNA concentration of 1 μM to exclude the higher structures/aggregates of tetrahedron. The white bars indicate 20 nm.

The mobility was the fastest for tetrahedron, followed by tetrapodna, then tetragon. These differences would be due to the structural differences of these DNA nanostructures, as all these DNA nanostructures consisted of 220 nucleotides. Figure 2B shows the PAGE analysis of these DNA nanostructures prepared at DNA concentration of 0.5–20 μM . A significant fraction of tetrahedron remained in the well, suggesting that it formed multimers or aggregates under these conditions at high DNA concentrations. The formation of tetrapodna, tetrahedron, and tetragon was confirmed using AFM. The PAGE analysis of DNA nanostructures for AFM experiments was shown in Figure 3. A DNA frame prepared using the DNA origami method was used to immobilize the DNA nanostructures for imaging. In addition, two of the four ODNs for each DNA nanostructure were extended to be immobilized to the frame. Figure 4 shows the AFM images of tetrapodna, tetrahedron, and tetragon immobilized to the DNA frame. AFM images with large area were shown in Figure 5.



Figure 5. AFM images of tetrapodna, tetrahedron, and tetragon. The white rectangles with a window represent DNA origami frames.

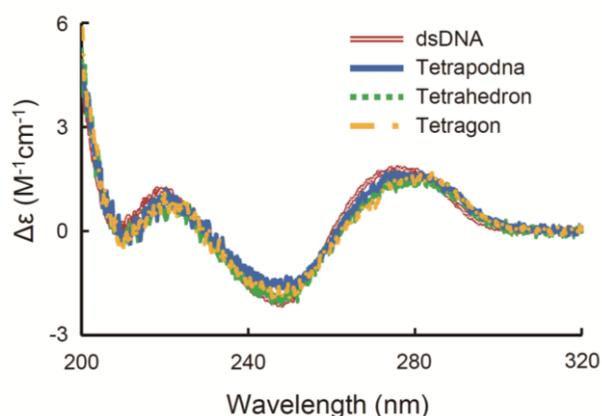


Figure 6. CD spectra of tetrapodna, tetrahedron, and tetragon. CD spectra were recorded using a JASCO-820 type spectropolarimeter. To facilitate comparisons, the background of CD spectra was subtracted.

The images clearly show that all of the preparations had different structures from one another. Tetrapodna was structured with four pods extruding from the center, the structural characteristics of which were almost identical to those of a larger tetrapodna consisting of 90-base ODNs²⁴. The AFM image of tetragon shows a rectangular shape as designed. On the other hand, the image for tetrahedron shows a triangular structure that was more complicated than the others.

I-1-3-b. T_m and apparent size of DNA nanostructures

The thermal stabilities of tetrapodna, tetrahedron, and tetragon were examined by measuring the T_m. Table 2 summarizes the T_m values in TE buffer containing 50 mM magnesium chloride. Tetrapodna had the highest T_m, followed by tetragon. The apparent sizes of these DNA nanostructures, measured by dynamic light scattering, are also listed in Table 2. All preparations were about 8 nm in diameter.

Table 2. T_m and apparent size of tetrapodna, tetrahedron, and tetragon.

DNA	T _m (°C)	Size (nm)
Tetrapodna	77.4	7.4 ± 0.8
Tetrahedron	68.3	8.5 ± 0.9
Tetragon	73.0	8.4 ± 1.0

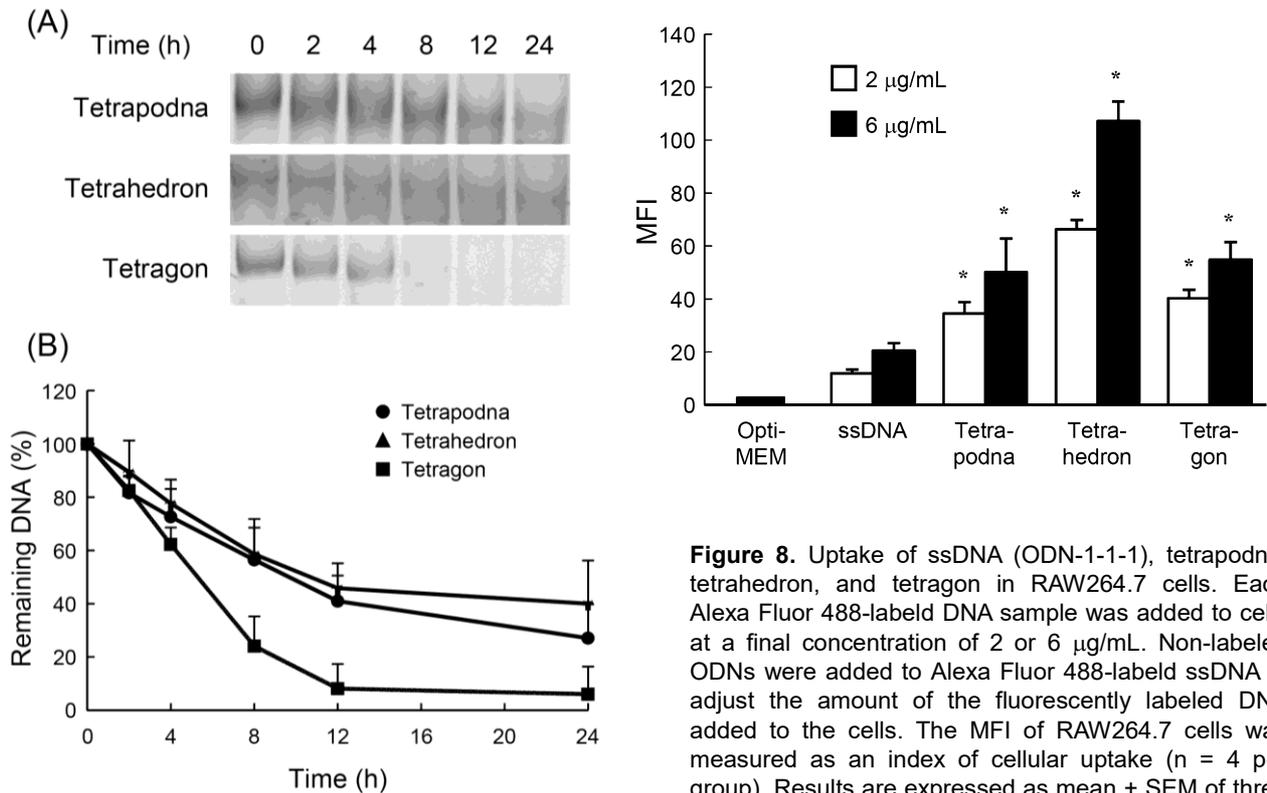


Figure 7. Degradation of tetrapodna, tetrahedron, and tetragon in 50% non-heat-inactivated FBS. (A) Each sample incubated at 37°C for indicated times was run on 9% polyacrylamide gel and stained with SYBR Gold. PAGE results are representative of five independent experiments. (B) The density of full-length DNA bands was quantitatively evaluated using the Multi Gauge software. The remaining amounts of full-length DNA were plotted against the incubation time. Results are expressed as mean + SEM of five independent experiments.

Figure 8. Uptake of ssDNA (ODN-1-1-1), tetrapodna, tetrahedron, and tetragon in RAW264.7 cells. Each Alexa Fluor 488-labeled DNA sample was added to cells at a final concentration of 2 or 6 µg/mL. Non-labeled ODNs were added to Alexa Fluor 488-labeled ssDNA to adjust the amount of the fluorescently labeled DNA added to the cells. The MFI of RAW264.7 cells was measured as an index of cellular uptake (n = 4 per group). Results are expressed as mean + SEM of three independent experiments. *P < 0.05 compared with ssDNA at the same concentration.

I-1-3-c. CD spectra of DNA nanostructures

Figure 6 shows the CD spectra of tetrapodna, tetrahedron, and tetragon. All samples examined had a positive peak near 280 nm and a negative peak near 240 nm, and there were no significant differences in the spectra. The spectra of the DNA nanostructures were similar to those of dsDNA, which exhibited B-form under the conditions examined.

I-1-3-d. Stability of DNA nanostructures in serum

Figure 7A shows the PAGE analysis of the DNA nanostructures incubated in solution containing 50% non-heat-inactivated FBS. The bands of the DNA nanostructures weakened with time in all cases. In addition, the bands shifted down with time because of the degradation of the DNA nanostructures. Figure 7B shows the time-courses of the remaining DNA after densitometric analysis of the gels. This analysis indicated that tetragon was quickly degraded with time compared with the others.

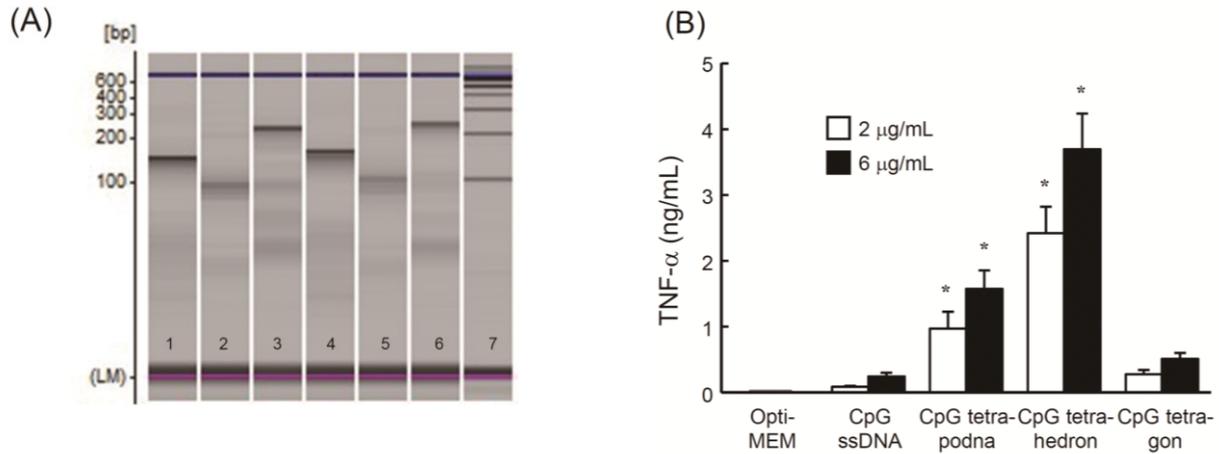


Figure 9. Preparation of CpG tetrapodna, CpG tetrahedron, and CpG tetragon and TNF- α release after addition of CpG DNAs to RAW264.7 cells. (A) Aliquots of tetrapodna, tetrahedron, and tetragon with (75 ng) or without CpG DNA (68 ng) prepared at 1 μ M DNA were run on a MultiNA microchip electrophoresis system. Lane 1, tetrapodna; lane 2, tetrahedron; lane 3, tetragon; lane 4, CpG tetrapodna; lane 5, CpG tetrahedron; lane 6, CpG tetragon; lane 7, 100-bp DNA ladder. (B) Each DNA sample was added to cells at a final concentration of 2 or 6 μ g/mL, and the concentrations of TNF- α in the supernatant were measured at 8 h ($n = 4$ per group). Non-CpG ODNs were added to CpG ssDNA to adjust the amount of the CpG DNA added to the cells. Results are expressed as mean + SEM of four independent experiments. * $P < 0.05$ compared with ssDNA at same concentration.

I-1-3-e. Uptake of Alexa Fluor 488-labeled DNA nanostructures in RAW264.7 cells

Figure 8 shows the MFI of RAW264.7 cells after addition of Alexa Fluor 488-labeled DNA samples. RAW264.7 cells with added Alexa Fluor 488-labeled tetrahedron showed the highest MFI value, followed by Alexa Fluor 488-labeled tetrapodna, tetragon, and ssDNA (ODN-1-1-1) in this order. The MFI values increased with increasing DNA concentration from 2 to 6 μ g/mL in all the cases.

I-1-3-f. TNF- α release from RAW264.7 cells after addition of CpG DNA nanostructures

Figure 9A shows the MultiNA analysis of tetrapodna, tetrahedron, and tetragon with an extending 25-base long sequence containing a CpG motif (Figure 10 shows the PAGE analysis of these DNA nanostructures). Each DNA nanostructure containing CpG DNA was named as CpG tetrapodna, CpG tetrahedron, and CpG tetragon, respectively. Figure 9B shows the amount of TNF- α released from RAW264.7 cells after addition of CpG ssDNA (CpG ODN-1-1-1), CpG tetrapodna, CpG tetrahedron, or CpG tetragon. Compared with CpG ssDNA, all of the CpG DNA nanostructures induced high amounts of TNF- α . CpG tetrahedron induced the largest amounts of TNF- α , followed by CpG tetrapodna. CpG tetragon induced much less TNF- α release from RAW264.7 cells than

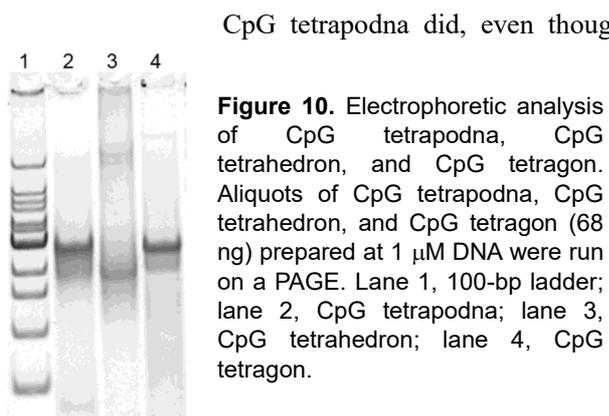


Figure 10. Electrophoretic analysis of CpG tetrapodna, CpG tetrahedron, and CpG tetragon. Aliquots of CpG tetrapodna, CpG tetrahedron, and CpG tetragon (68 ng) prepared at 1 μ M DNA were run on a PAGE. Lane 1, 100-bp ladder; lane 2, CpG tetrapodna; lane 3, CpG tetrahedron; lane 4, CpG tetragon.

CpG tetrapodna did, even though there were no significant differences in the uptake between Alexa Fluor 488-labeled tetrapodna and tetragon (Figure 8). Tetrapodna, tetrahedron, or tetragon containing no CpG motifs induced little TNF- α release when added to RAW264.7 cells (data not shown).

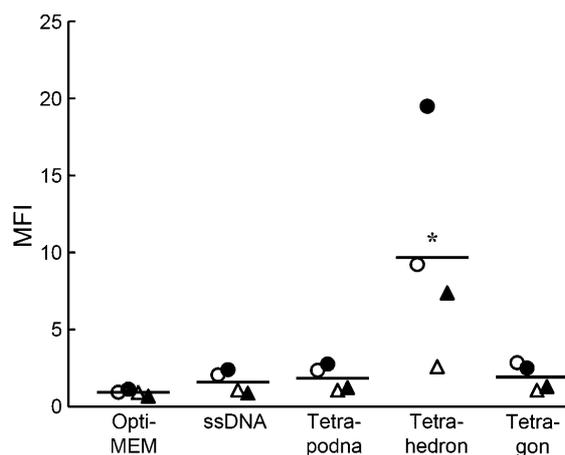


Figure 11. Uptake of ssDNA (ODN-1-1-1), tetrapodna, tetrahedron, and tetragon in human PBMCs. Each Alexa Fluor 488-labeled DNA sample was added to cells at a final concentration of 6 $\mu\text{g}/\text{mL}$. Non-labeled ODNs were added to Alexa Fluor 488-labeled ssDNA to adjust the amount of the fluorescently labeled DNA added to the cells. The MFI of PBMCs was measured as an index of cellular uptake ($n = 4$ per group). Each symbol represents a mean of a single experiment, and each experiment is identified with different symbols. Bars represent the mean of four independent experiments. * $P < 0.05$ compared with ssDNA.

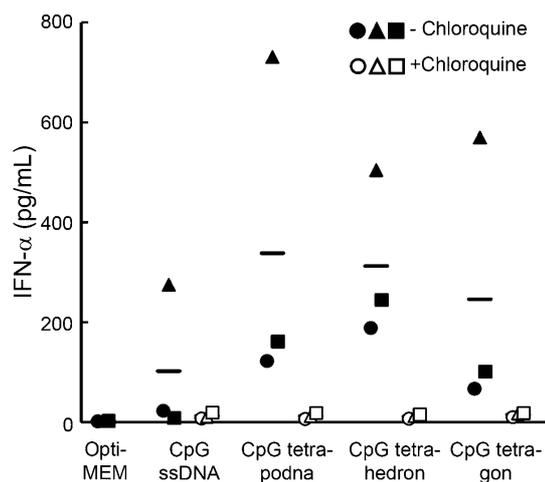


Figure 12. IFN- α release after addition of CpG DNAs to human PBMCs with or without treatment with 100 μM chloroquine. Each DNA sample was added to cells at a final concentration of 6 $\mu\text{g}/\text{mL}$, and the concentrations of IFN- α in the supernatant were measured at 24 h ($n = 4$ per group). Non-CpG ODNs were added to CpG ssDNA to adjust the amount of the CpG DNA added to the cells. Each symbol represents a mean of a single experiment, and each experiment is identified with different symbols. Bars represent the mean of three independent experiments.

I-1-3-g. DNA uptake and IFN- α release after addition of DNA nanostructures to human PBMCs

Figure 11 shows the MFI of human PBMCs after addition of Alexa Fluor 488-labeled DNA samples. PBMCs with added Alexa Fluor 488-labeled tetrahedron showed the highest MFI value. The MFI values of PBMCs added with either Alexa Fluor 488-labeled ssDNA (ODN-1-1-1), tetrapodna, or tetragon were comparable to one another. Figure 12 shows the amount of IFN- α released from RAW264.7 cells after addition of CpG ssDNA (CpG ODN-1-1-1), CpG tetrapodna, CpG tetrahedron, or CpG tetragon in the absence or presence of chloroquine. All of the CpG DNA nanostructures induced high amounts of IFN- α compared with CpG ssDNA (CpG ODN-1-1-1). IFN- α release was greatly inhibited by chloroquine.

I-1-4 Discussion

The versatility of DNA nanotechnology makes it possible to design DNA nanostructures of any shape because the number of ODNs as well as their sequence and length can be arbitrarily selected. This is one of the major advantages of DNA nanotechnology, and it was used in the present study to construct and compare three different DNA nanostructures. However, a set of ODNs used for a DNA nanostructure cannot be used to construct other nanostructures, and a different set of ODNs should be prepared for each DNA nanostructure. This difference should be kept in mind when the structural properties of DNA nanostructures are compared with one another. In this study, one of the four ODNs was used for all the three DNA nanostructures to minimize the differences in the sequence of the ODNs. In addition, the GC content, which is a key parameter determining the thermal stability of

the structures, was adjusted to be comparable among the three DNA nanostructures between 45% and 47%. Finally, the lack of significant TNF- α release from cells added with any DNA nanostructure containing no CpG motifs indicated that TNF- α release after addition of the CpG DNA nanostructures could be attributed to the CpG motif.

A set of ODNs reported by Li et al. was selected for the comparison because our preliminary studies showed that tetrahedron had much lower flexibility in design than tetrapodna. For example, tetrahedron was not obtained when four 43-mer ODNs were used (Ohtsuki et al., unpublished results), whereas tetrapodna could be formed using four ODNs from 36 to 90-mer ODNs²⁴. In addition, the present study revealed that high ODN concentrations resulted in the formation of multimers or aggregates except for tetrapodna, greatly limiting the conditions for preparing the DNA nanostructures for comparison, even though preparation efficiency is one of the most important parameters as far as biopharmaceutical applications are concerned.

Structures other than those examined in this study can also be designed using four 55-mer ODNs. A simpler structure is a linear one partly or fully containing double-stranded DNA. The structural properties of such linear DNA would be similar to those of double-stranded DNA, and therefore, it was not included for comparison in this study. Other structures would share the structural properties with either tetrapodna (branched DNA), tetrahedron (three-dimensional DNA), or tetragon (rectangular or circular DNA), as far as symmetric structures are concerned. Therefore, the three DNA nanostructures would be reasonable for comparing the structural and biological properties of DNA nanostructures that were designable using four short ODNs.

The present study clearly demonstrated that tetrapodna was far the best DNA nanostructure in terms of both the preparation efficiency and biological activity. This would be, at least in part, attributed to its high thermal stability (Table 2). The lengths of the consecutive double-stranded parts in each structure were 27, 17, and 26 for tetrapodna, tetrahedron, and tetragon, respectively, and the long double-stranded parts could explain the high T_m value of tetrapodna. The T_m values of the incomplete structures consisting of three out of four ODNs were 73°C, 68°C, and 71°C for tetrapodna, tetrahedron, and tetragon, respectively. In addition, the T_m values of the 27-mer double-stranded DNA identical to a branch of tetrapodna and 26-mer double-stranded DNA identical to a side of tetragon were 72°C and 70°C, respectively. These results strongly indicated that the complete structure of tetrapodna was more thermally stable than the incomplete ones. This unique property is a reason for the very high preparation efficiency of tetrapodna, even at high DNA concentrations. In contrast, the others were not stabilized by the formation of complete structures, which led to the formation of multimers/aggregates at high DNA concentrations.

The above discussions on the thermal stability suggested that it is difficult to re-design tetrahedron with high preparation efficiency even at high DNA concentrations. Li et al. reported that tetrahedron could be obtained by self-assembly of four ODNs, but the authors prepared tetrahedron at a low DNA concentration of 1 μM ³². As demonstrated in the present study, increasing the DNA concentration reduced the preparation efficiency

of tetrahedron. Any approach to avoid the oligomerization of tetrahedron would greatly expand its usefulness as delivery vehicles to immune cells.

DNA nanostructures, including tetrahedron, were considered to be monomer under the experimental conditions examined, but the possibility that they form multimers or aggregates at their interaction with cells cannot be excluded at this moment. Therefore, there is still a possibility that the efficient cellular uptake of tetrahedron is due to its more efficient formation of multimers or aggregates than the others.

Previous study from my laboratory on the interaction of polypodnas with immune cells, including RAW264.7 cells and mouse bone marrow-derived dendritic cells, indicated that DNA assemblies with bulky structures were more efficiently taken up by the cells compared with ones with sparse structures²²⁻²⁵. A previous study reported that longer DNA was better than short ones in terms of cellular uptake²⁴. Several receptors or membrane proteins have been reported to be involved in the cellular uptake of DNA^{35,36}, although no consensus applicable to various cells has been reached yet. However, the results of the cellular uptake of DNA preparations suggested that the interaction of DNA with the cell surface at multiple sites facilitated its cellular uptake. The present study showed that tetrahedron was better than tetrapodna in terms of the uptake by RAW264.7 cells (Figure 8). The formation of oligomers might be involved in the efficient uptake of tetrahedron, even though tetrahedron was prepared under conditions where multimers were not easily formed (Figure 2A, lane 9). The reason for this efficient cellular uptake of tetrahedron requires further investigation.

The experimental results obtained with human PBMCs showed large variations compared to those with RAW264.7 cells, probably because of intraindividual and/or interindividual variability in the property of the cells. Generally speaking, however, the results were comparable to those with RAW264.7 cells, suggesting that the DNA nanostructures examined in this study, especially CpG tetrapodna and CpG tetrahedron, induce cytokines, including IFN- α , in humans more efficiently than CpG ssDNA. The substantial inhibition of IFN- α release from human PBMCs by chloroquine, an inhibitor of endosomal TLR signaling, strongly suggests that the TLR9 is involved in the IFN- α release from the cells after addition of CpG tetrapodna, CpG tetrahedron or CpG tetragon. In contrast, the uptake of Alexa Fluor488-labeled tetrapodna and tetragon by human PBMCs was comparable to that of Alexa Fluor488-labeled ssDNA, which was different from the results obtained using RAW264.7 cells (Figure 11). A possible explanation on this difference is that the population of cells that can be responsible for stimulation with CpG DNA in PBMCs, i.e., plasmacytoid dendritic cells, is quite low³⁷. Higher uptake of Alexa Fluor488-labeled tetrahedron than the others may indicate that tetrahedron is efficiently taken up not only by plasmacytoid dendritic cells but by other cells in PBMCs.

The present study clearly showed that tetrapodna had the highest thermal stability, best formation efficiency, and CpG tetrapodna possessed high potency to induce TNF- α release from RAW264.7 cells and IFN- α release from PBMCs. Despite efficient cellular uptake and TNF- α release, the low formation efficiency of tetrahedron greatly limited its application as a delivery vehicle of immunostimulatory DNAs. CpG tetragon was the least efficient in terms of TNF- α release. Taken together, I conclude that tetrapodna is the best assembly as

delivery vehicles for immunostimulatory nucleic acids to immune cells, and that tetrahedron can be another useful assembly for cellular delivery if its preparation yield is improved³⁸.

Chapter I

Section 2

Effect of the structural properties of tripod-like structured DNA on their interaction with macrophage-like RAW264.7 cells

I-2 Summary

In Section 1, it was found that there was correlation between the structural properties and cellular uptake of nanostructured DNA. However, it was not clear which structural features of the three-dimensional structure are important for the cellular uptake. The aim of this section was to describe what kind of properties increase the uptake of nanostructured DNA and cytokine release from the immune cells upon uptake of the nanostructure DNA. To solve this question, I selected tripod-like structured DNA (tripodna) as a three-dimensional nanostructured DNA that can be easily evaluated and the effect of the structural featured of tripodna on the interaction of tripodna and the cells was evaluated. In this section, I designed several types of tripodna and DNA assemblies consisting of double-stranded DNA or nick double-stranded DNA.

A previous study from my laboratory reported that hexapod-like structured DNA had efficient interaction with immune cells compared with tripod-like structured DNA, or tetrapod-like structured DNA preparations of same length of ODNs²⁴. The effect of size on cell uptake has been described so far³⁹⁻⁴¹, but details on 3-D recognition at the same molecular weight are required. In the Section 1, I revealed that the structural property of nanostructured DNA could affect the interaction with immune cells. Then, I selected tripod-like structured DNA to examine the details on the structural properties of nanostructured DNA required for the cellular uptake.

A previous study reported that DNA with a neck had a low melting temperature (T_m) compared with double stranded DNA without neck structures⁴². Experiments using asymmetric tripodna preparations showed that the T_m was a function of the length of the neck and using a length half that of the other parts resulted in a minimal T_m value. The presence of a short neck would force the double stranded DNA to bend, but it would not help the structure to be stabilized because the length is too short. Taken together, the T_m of polypodna is a complicated function of the bend angle and the length of each pod.

TLR9 recognition of CpG DNA occurs after endocytosis and the dissociation of double-stranded DNA into single strands depends on the endosomal environments. In this study, double-stranded DNA with a nick made with three ODNs was also used in addition to completely complementary double-stranded DNA, because the former seems to be more easily dissociated into single strands than the latter.

In both studies of cellular uptake and cytokine release, tripodna with a perfect structure had the highest values in terms of cellular uptake and subsequent cytokine production.

In this section, I concluded that bent DNA structures are efficiently recognized by RAW264.7 cells than linear ones.

Chapter I

Section 3

Elucidation of the mechanism of increased activity of immunostimulatory DNA by the formation of polypod-like structure.

I-3 Summary

In Section 1 and 2, the results suggested that nanostructured DNA including polypod-like structured DNA is useful for delivery of nucleic acid drugs to macrophage-like RAW264.7 cells. However, for clinical use, it is also necessary to evaluate their characteristics by using cells other than target cells. Moreover, the uptake pathways of nanostructured DNA need to be clarified. For this purpose, I selected two types of polypodna: tripodna and hexapodna. I examined their uptake in several types of immune and non-immune cells. Then, I also tested other type of nanostructured DNA including tetrapodna, tetrahedron, and tetragon same as Section 1 to elucidate the influence of structural properties.

For clinical use of nucleic acid drugs, it is necessary to consider the uptake by various types of cells, including immune cells and non-immune cells. Several reports suggested that nanostructured DNAs are useful for the delivery of nucleic acid to immune cells. In this section, I selected RAW264.7 cells and DC2.4 cells as immune cells, and MAEC cells, C166 cells, NIH3T3 cells, C2C12 cells, and HEK-Blue hTLR9 cells as non-immune cells. Nanostructured DNAs were more efficiently taken up by immune cells than by non-immune cells. This result suggested that nanostructured DNAs have potential of selective delivery of nucleic acids to immune cells.

Clathrin-mediated endocytosis, an well-known endocytic pathways, is caused by ligand binding to cell surface receptors, and endosomal vesicles formed by clathrin are taken up by cells⁴³⁻⁴⁵. Results suggested that nanostructured DNAs were efficiently taken up by RAW264.7 cells via clathrin-mediated endocytosis, and they were hardly taken up via endocytic pathways by NIH3T3 cells.

There are two general intracellular pathways for uptake of nanosized molecules. Large molecules than 250 nm are taken up by cells via phagocytosis, and small molecules are taken up via pinocytosis⁴⁶. Pinocytosis includes micropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-/caveolae-independent endocytosis⁴⁷. In phagocytosis pathway and micropinocytosis pathway, nanosized molecules taken up by cells are transferred directly to the lysosome, while molecules taken up by cells via clathrin-mediated endocytosis are transported to endosome. In present section, nanosized structured CpG DNAs were effectively recognized by endosomal TLR9. These strongly suggest that clathrin-mediated endocytosis would be involved in cellular uptake of nanostructured DNAs. However, further investigation about endocytic receptors is required.

Taken together, uptake of nanostructured DNAs in immune cell was more efficient than non-immune cells, and calthrin-mediated endocytosis was involved in the uptake process of nanostructured DNAs in RAW264.7 cells.

Chapter II

Design of DNA-based delivery systems to immune cells using DNA origami or DNA supramolecules

The results in Chapter I suggest that tetrahedral DNA or bend DNA could efficiently interact with immune cells, while there is little information about structural properties. Then, I hypothesized that the structural rigidity of nanostructured DNAs would be a key for their interaction with immune cells. In Chapter II, I used two experimental methods to elucidate involvement of structural rigidity or structural compactness in cellular uptake of nanostructured DNA by immune cells.

In Section 1, I used DNA origami technology to elucidate the involvement of structural rigidity in the cellular uptake of nanostructured DNA. DNA origami technology is useful for create nanoscale shapes and patterns. Then, I designed various rectangular DNAs with varying numbers of staple strands. Rectangular DNA with fewer staples could have more structural flexibility than rectangular DNA with full staples. The cellular uptake of these nanostructured DNAs was evaluated.

In Section 2, DNA supramolecules formed by hydrophobic interaction were designed. To elucidate the involvement of structural compactness, I designed RCA products and cholesterol modified RCA products with different compactness.

In this chapter, the results are presented in the following two sections.

Chapter II

Section 1

Folding of single-stranded circular DNA into rigid rectangular DNA origami accelerates its cellular uptake

II-1 Summary

DNA origami technology is useful for create nanosized DNA assemblies^{17,48,49}. These DNA origami-based nanostructures have been applied as sensors for single nucleotide polymorphism (SNP) and RNA sequences^{50,51}.

It was found that the increased activity of CpG DNA by constructing nanosized DNA assemblies was attributed to the increased cellular uptake. My laboratory previously demonstrated that the cellular uptake of single-stranded, double-stranded, and branched, polypod-like structured DNAs was dependent on their structural complexity²⁴. My study in Chapter I using tetrapod-like structured DNA (tetrapodna), tetrahedral DNA (tetrahedron), and tetragonal DNA (tetragon), all of which were prepared using four ODNs of the same length, also showed that the uptake of these nanostructured DNA by RAW264.7 cells was quite different from one another³⁸. Then, I hypothesized that structural rigidity could be a key for efficient delivery of nucleic acid drugs to immune cells. Although there is little information about the relationship between the structural rigidity and cellular uptake of DNA nanostructures, it can be speculated, based on their possible structural flexibility, that tetrahedron may be more rigid than the others. This difference in flexibility may lead to differences in their cellular uptake.

To prove this hypothesis, nanostructured DNAs with similar shape but different flexibility were preferable. In the present section, a rectangular DNA (RecDNA) was designed with a scaffold DNA and 50 staples, and ones with fewer staples were also prepared as more flexible ones. The cellular uptake of these DNA nanostructures was evaluated after addition to RAW264.7 cells.

Cellular uptake was highest in RecDNA, and nanostructured DNA with fewer staples, which are probably more flexible, showed lower uptake.

Taken together, the present section showed that the cellular uptake of RecDNA, the rectangular DNA with full staples, was more efficient than DNA nanostructure with fewer staples.

Chapter II

Section 2

Development of DNA supramolecules formed by hydrophobic interaction for the effective delivery of CpG DNA

II-2 Summary

My laboratory previously reported that various DNA nanostructures can be prepared using three or more oligodeoxynucleotides (ODNs). The DNA nanostructures were efficiently taken up by mouse macrophage-like RAW264.7 cells, and increased the immunostimulatory activity of CpG DNA. Therefore, DNA nanostructures can be used to efficiently deliver CpG DNA to immune cells^{22,24,38}.

In Chapter I, Section 1, I revealed that tetrahedral DNA had the most efficient interaction with immune cells. In Chapter II, Section 1, I also concluded that rectangular DNAs with many staples were more efficiently taken up by RAW264.7 cells. These results imply that compactness of DNA nanostructures may be related to the cellular uptake of DNA nanostructures.

Rolling circle amplification (RCA) is a simple and efficient isothermal enzymatic process in which a circularized template DNA is amplified by DNA polymerase. RCA is able to produce long single stranded DNA (ssDNA) composed of multiple tandem repeats that are complementary to a circular DNA template^{52,53}. In this section, I tried to prepare amphiphilic DNA supramolecules composed of CpG DNA-containing RCA products added with hydrophobic segments for the efficient delivery of CpG DNA.

I developed CpG DNA delivery carrier composed of RCA products (RCA_{pro}) and complementary chol-DNAs. RCA products were hybridized with chol-DNA (RCA_{pro}-chol) via DNA-DNA base pairing and formed the supramolecules probably because of hydrophobic cholesterol groups. I hypothesized that RCA_{pro}-chol was more compact than RCA_{pro}.

TEM observation revealed that RCA_{pro}-chol showed more spherical shape than RCA and RCA_{pro}. In addition, dynamic light scattering analysis revealed that the size of RCA_{pro}-chol was approximately 5-fold smaller in diameter than RCA_{pro}. The uptake of RCA products in RAW264.7 cells, was increased by hybridization with chol-DNA. Moreover, the DNA supramolecules significantly induced TNF- α release from RAW264.7 cells. These results suggested that spherical shape with structural compactness is a key for cellular uptake of DNA.

In conclusion, the present section shows that RCA_{pro}-chol that formed supramolecules delivered CpG DNA to RAW264.7 cells and enhanced the immunostimulatory activity of CpG DNA. The result of TEM showed that RCA_{pro}-chol was more compact than RCA_{pro}, and suggested that structural compactness could be a key for their interaction with immune cells. These results indicate that the DNA supramolecules are suitable tool for delivering CpG DNA to immune cells.

Chapter III

Elucidation of cell surface receptors involved in the cellular uptake of nanostructured DNA

III-1 Introduction

Various classes of nucleic acid drugs are being marketed or under development. Attention must be paid to toxicity issues during the development of nucleic acid drugs⁵⁴. Nucleic acid drug candidates have several toxicity issues, including off-target effects, immune stimulation, hematotoxicity, hepatotoxicity, and nephrotoxicity. Immune stimulation occurs when toll-like receptors (TLRs) recognize DNA or RNA. Several reports have suggested that certain small interfering RNA molecules cause immune stimulation via TLRs⁵⁵⁻⁶¹. Therefore, it is clearly important to evaluate unexpected TLR-mediated immune stimulation in the development of nucleic acid drug candidates.

TLR9 is the only TLR that recognizes DNA. Its ligand is a DNA molecule containing an unmethylated cytosine-phosphate-guanine (CpG) motif, i.e., CpG DNA. Because bacterial and viral DNAs contain many CpG motifs, TLR9-mediated responses form part of the self-defense system against the invasion of such pathogens^{7,62,63}. TLR9 is expressed in the endosomes of various mammalian cells, including plasmacytoid dendritic cells and B-cells⁶⁴. After cellular uptake and sorting into endosomes, CpG DNA binds to TLR9 and induces the release of proinflammatory cytokines, which then activate innate immunity^{10,65}. It is expected that such responses can be exploited to treat cancer, infection, and allergic diseases^{11,12}. I have previously reported that nano-construction of CpG DNA is a unique and promising method of increasing the immunostimulatory activity of CpG DNA³⁸. Studies using a series of polypod-like structured DNAs demonstrated that the cellular uptake of CpG DNA was significantly increased by its incorporation into nanostructured DNAs^{24,25,66}. However, the mechanisms involving the structure-dependent uptake of DNA require further elucidation. To develop efficient delivery systems, interaction of nanostructured DNA and immune cells needs to be elucidated and optimized.

The endosomal localization of TLR9 indicates that membrane protein(s) other than TLR9 are responsible for the cellular uptake of DNA. So far, several DNA receptors have been reported. These include macrophage scavenger receptor-1 (MSR1, SR-A, and CD204), α M β 2 (MAC-1), advanced glycosylation end product-specific receptor (AGER), membrane-associated nucleic acid-binding protein (MNAB), mannose receptor-1 (MRC1), and lymphocyte antigen 75 (DEC-205)^{35,36,67-70}. However, in most cases their contribution to DNA uptake has been examined using phosphorothioate (PS) DNA, which non-specifically binds to cell membranes more strongly than natural phosphodiester (PO) DNA. The results obtained with PS DNA cannot be used to estimate the contribution of DNA receptors in the cellular responses to nucleic acid drug candidates. Msr1, which mediates the endocytosis of negatively charged molecules such as acetylated low-density lipoprotein (LDL) and oxidized LDL, is reportedly involved in the uptake of PO DNA by macrophages⁶⁷. Therefore, Msr1-mediated DNA uptake could be involved in TLR-mediated immune stimulation by nucleic acid drug candidates.

HEK-Blue TLR cells are commercially available and can be used for the analysis of the immunological properties of various TLR ligands⁷¹. HEK-Blue hTLR9 cells respond to PS CpG DNA and release secreted embryonic alkaline phosphatase (SEAP)⁷². However, my preliminary studies have shown that PO CpG DNA induces very little SEAP release from HEK-Blue hTLR9 cells. Because HEK293 cells, which constitute the parent

cell line of HEK-Blue TLR9 cells, are non-immune cells, they do not express any DNA receptors on their cell membranes. Therefore, I hypothesized that low cellular uptake of CpG DNA by HEK-Blue hTLR9 cells might explain the weak or absent response to PO CpG DNA. Therefore, in the present chapter, I sought to establish cell lines that respond to both PS and PO CpG DNAs. Such cells would be useful for the screening of nucleic acid drug candidates with diverse physicochemical properties.

I revealed that nanosized DNA assemblies are useful for delivery of nucleic acid drugs to immune cells in Chapter I and Chapter II. I also elucidated that structural rigidity or structural compactness could be a key for efficient delivery of nucleic acid drugs to immune cells via cellular uptake receptor. Then, I also hypothesized that HEK-Blue hTLR9 cells expressing cell surface receptor candidates is useful for elucidate the involvement in cellular uptake of nanostructured DNA to immune cells.

To this end, I transduced HEK-Blue hTLR9 cells with human hMSR1 to obtain HEK-Blue hTLR9/hMSR1 cells in the hope that the transfection of the *MSR1* gene to HEK-Blue hTLR9 cells would increase the uptake of PO DNA. I first evaluated the effect of transfection of the *MSR1* gene on the cellular uptake of DNA. I then determined whether HEK-Blue hTLR9/hMSR1 cells respond to both PS and PO CpG DNAs. I selected phosphorothioate CpG2006 (PS CpG2006), a single-stranded PO CpG DNA (ssCpG), and a tetrapod-like structured DNA containing the ssCpG (tetraCpG) as model TLR9 ligands. HEK-Blue hTLR9 cells and HEK-Blue hTLR7 cells were also used for the analysis of cellular responses to CpG DNA.

III-2 Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). Sodium chloride, sodium hydrogen phosphate, sodium bicarbonate, potassium chloride, glucose, sodium dodecyl sulfate (SDS) methanol, and WIDE-VIEW Prestained Protein Size Marker III were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Tris was obtained from Nacalai Tesque (Kyoto, Japan). Blastocidin, zeocin, normocin, CL264, and HEK-Blue detection reagents were purchased from InvivoGen (San Diego, CA, USA). Opti-modified Eagle's medium (Opti-MEM) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). A 100-base pair (bp) DNA ladder was purchased from Takara Bio (Otsu, Japan). All other chemicals were of the highest grade available and were used without further purification.

Cell culture

HEK-Blue hTLR7 and HEK-Blue hTLR9 cells were obtained from InvivoGen. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 0.2% sodium bicarbonate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 10 mg/mL blastocidin, 100 mg/mL zeocin, and 50 mg/mL normocin at 37°C in humidified air containing 5% CO₂ as per the manufacturer's instructions.

Plasmid DNA

Plasmid pcDNA3.1 was purchased from Thermo Fisher Scientific Inc. A plasmid vector encoding human macrophage scavenger receptor-1 (human MSR1, hSR-A, hCD204) was constructed by insertion of the FLAG-tagged MSR1 fragment amplified by polymerase chain reaction (PCR) from a cDNA clone of human MSR1 (GE Healthcare UK Ltd., Buckinghamshire, England) into the multi-cloning site of pcDNA3.1.

Transfection of hMSR1-expressing plasmid DNA in HEK-Blue cells

HEK-Blue hTLR7 and HEK-Blue hTLR9 cells were cultured in 75-cm² tissue culture flasks, and were transfected with a pcDNA3.1 vector encoding MSR1 or an empty pcDNA3.1 vector using Lipofectamine 2000 (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. After 20 h of incubation, the cells were used as HEK-Blue hTLR7/hMSR1 and HEK-Blue hTLR9/hMSR1 cells. The cells transfected with empty pcDNA3.1 vector were used as mock controls.

Western blotting of hMSR1 in HEK-Blue hTLR cells

The cells were lysed in a lysis buffer (PicaGene Dual Sea Pansy Luminescence Kit, Toyo Ink, Tokyo, Japan), and the cell lysates were reduced by the addition of dithiothreitol to 100 mM. A fraction of the cell lysate (7 µg protein) was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride transfer membrane (Immobilon-P; Merck Millipore Ltd, Darmstadt, Germany). The membrane was then blocked in Blocking One (Nacalai Tesque, Kyoto, Japan). The membrane was incubated with anti-hMSR1 antibody (R&D Systems, Minneapolis, MN, USA) for 1 h at 20–22°C. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (Thermo Fisher Scientific Inc.) for 1 h at room temperature. Protein bands were detected by chemiluminescence using an Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Billerica, MA, USA).

Confocal microscopic detection of hMSR1 in HEK-Blue cells transduced with hMSR1

Untreated, mock-transfected, or *MSR1*-transfected HEK-Blue hTLR9 cells were seeded on a chamber slide at a density of 3×10^4 cells/well and then cultured for 24 h. The cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min, and washed again twice with PBS. The cells were then blocked with 20% FBS in PBS for 1 h. The cells were incubated with anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS in PBS for 1 h at room temperature, and then washed once. The cells were incubated with Alexa Fluor 488-labeled anti-mouse IgG antibody (Abcam Plc, Cambridge, UK) for 1 h at room temperature, and washed once. The cells were incubated with 600 nM 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) for 5 min at room temperature and washed once. The chamber was then removed and the slide was observed using a confocal microscope (A1R MP, Nikon Instech Co., Ltd., Tokyo, Japan) as previously reported⁷³.

Oligodeoxynucleotides

All oligodeoxynucleotides (ODNs) used were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The sequences of the ODNs used are presented in Table 3. Phosphodiester ODN-3-1, which contained a potent human CpG motif (GTCGTT), was used as single-stranded CpG DNA (ssCpG). ssCpG and three other phosphodiester ODNs were dissolved in an annealing buffer (TE buffer, 10 mM Tris-HCl, pH 8, 1 mM ethylenediaminetetraacetic acid, and 150 mM sodium chloride) and mixed in sterile water to produce a final concentration of 100 μ M for each ODN. The mixtures were then incubated at 95°C for 5 min and slowly cooled to 4°C using a thermal cycler to obtain tetrapodna containing ssCpG (tetraCpG). Phosphorothioate CpG2006 (PS CpG2006), a single-stranded B-type CpG DNA, was used as a positive control to induce SEAP release from the HEK-Blue hTLR9 cells. For cellular uptake experiments, ODN-3-1 labeled with Alexa Fluor 488 at the 5' end was purchased from Japan BioService Co., Ltd. (Saitama, Japan). Each sample was analyzed at room temperature by 6% PAGE. The DNA bands were visualized using SYBR Gold (Molecular Probes, Eugene OR, USA).

Uptake of DNA in HEK-Blue cells

Untreated, mock-transfected, or *MSRI*-transfected HEK-Blue hTLR9 cells were seeded onto 48-well plates at a density of 1×10^5 cells/well. Alexa Fluor 488-ssCpG or Alexa Fluor 488-tetraCpG diluted with 0.1 mL of Opti-MEM was then added to the cells. After 2 h incubation at 37°C, the cells were washed three times with 400 μ L of PBS and harvested. The fluorescence intensity of the cells was then determined by flow cytometry (Gallios Flow Cytometer; Beckman Coulter, Inc., CA, USA) using Kaluza software (version 1.0; Beckman Coulter), and the mean fluorescence intensity (MFI) was calculated. Similar experiments were carried out on the untreated, mock-transfected, and HEK-Blue hTLR7 cells.

SEAP release from HEK-Blue cells

The untreated, mock-transfected, or *MSRI*-transfected HEK-Blue hTLR9 cells were seeded onto 96-well plates at a density of 5×10^4 cells/well. PS CpG2006, ssCpG, or tetraCpG in HEK-Blue detection solution was added to the cells to produce a final concentration of 50 μ g/mL. After 20 h incubation at 37°C, the optical density (OD) of the samples was measured at a wavelength of 620 nm using a microplate reader. Similar experiments were carried out on untreated, mock-transfected, and HEK-Blue hTLR7 cells.

Inhibition of DNA uptake in HEK-Blue cells by anti-hMSR1 antibody

The untreated, mock-transfected, or *MSRI*-transfected HEK-Blue hTLR9 cells were seeded onto 48-well plates at a density of 1×10^5 cells/well. The cells were pretreated with anti-hMSR1 antibody or IgG1 isotype control (R&D Systems, Minneapolis, MN, USA) at a concentration of 2 μ g/mL for 1 h. After washing, the cells were treated with 2 μ g/mL Alexa Fluor 488-ssCpG or Alexa Fluor 488-tetraCpG diluted with 0.1 mL of Opti-MEM together with anti-hMSR1 antibody or IgG1 isotype control to produce a final concentration of 2

µg/mL. After 2 h incubation at 37°C, the cells were washed three times with 400 µL of PBS and harvested. The fluorescence intensity of the cells was determined by flow cytometry using Kaluza software, and the MFIs were calculated.

Statistical analysis

Differences were evaluated statistically by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer test for multiple comparisons and the Student’s t-test for two groups. P < 0.05 values were considered statistically significant.

Table 3. The sequences of the oligodeoxynucleotides (ODNs) for the DNA nanostructures.

Name	Sequences (5' → 3')	Length (mer)
ODN-1	<u>TCGTCGTTT</u> <u>TGTCGTTT</u> <u>TGTCGTT</u> TACATTCCTAAGTCTGA AACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA	80
ODN-1'	TACTATGGCGGCTCTTCTCGTGTAGCAAGCTGTAATGTT CAGACTTAGGAATGT	55
tetrapodna-2	TTACTATGGCGGCTCTTCTCGTGTAGCATAGTGTCGTTTTA TCACCAGGCAGTTG	55
tetrapodna-3	TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTT GACAGGTCATCAGCC	55
tetrapodna-4	TGGCTGATGACCTGTCAAGATTCCCACGAGCTGTAATGTT TCAGACTTAGGAATG	55
PS CpG2006	T*C* <u>G</u> *T*C* <u>G</u> *T*T*T*T* <u>G</u> *T*C* <u>G</u> *T*T*T*T* <u>G</u> *T*C* <u>G</u> *T*T	24

All ODNs have a phosphodiester backbone. The asterisks (*) indicate the positions of phosphorothioate (PS) modifications. The CpG motif (GTCGTT) is underlined.

III-3 Results

III-3-a. Establishment of HEK-Blue hTLR9/hMSR-1 cells

Figure 13A shows the results of western blotting analysis of the cell lysates using anti-hMSR1 antibody. The lysate of HEK-Blue hTLR9/hMSR1 cells revealed a band of approximately 75 kDa, which corresponded to the FLAG-tagged hMSR1. The band was not detected in the lysates of the untreated or mock-transfected HEK-Blue hTLR9 cells, indicating that FLAG-tagged hMSR1 was expressed in the HEK-Blue hTLR9/hMSR1 cells. I examined the localization of hMSR1 in the HEK-Blue hTLR9 cells using confocal microscopy. Figure 13B presents confocal microscopy images of untreated, mock-transfected, and MSR1-transfected HEK-Blue hTLR9 cells. The Alexa Fluor 488-labeled anti-FLAG antibody was bound to the cell surface of the HEK-Blue

hTLR9/hMSR1 cells. However, there was little fluorescence in the untreated or mock-transfected cells. These results indicate that hMSR1 localizes at the cell surface of the HEK-Blue hTLR9/hMSR1 cells.

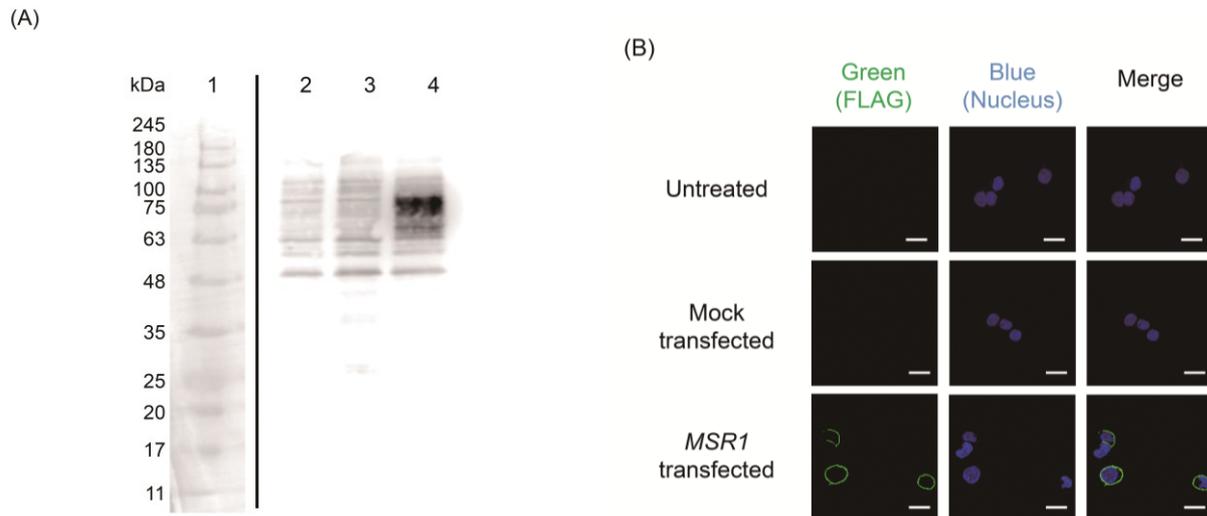


Figure 13. Confirmation of hMSR1 expression in HEK-Blue hTLR9 cells. (A) hMSR-1 protein was detected by western blotting using anti-hMSR1 antibody. Bright field (lane 1) and chemiluminescence (lanes 2-4) images were shown. Lane 1, protein size marker; lane 2, untreated HEK-Blue hTLR9 cells; lane 3, mock-transfected HEK-Blue hTLR9 cells; lane 4, HEK-Blue hTLR9/hMSR1 cells. (B) Confocal microscopy images of untreated, mock-transfected, or *MSR1*-transfected HEK-Blue hTLR9 cells immunostained with anti-FLAG antibody and Alexa Fluor-488 conjugated anti-mouse IgG1 antibody. Scale bar, 20 μ m.

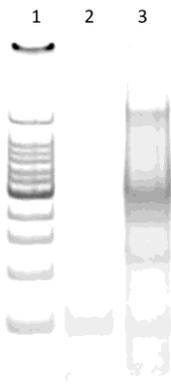


Figure 14. Electrophoretic analysis of ssCpG and tetraCpG. Aliquots of ssCpG and tetraCpG were run on 6% PAGE at room temperature. Lane 1, 100 bp ladder; lane 2, ssCpG; lane 3, tetraCpG.

III-3-b. Evaluation of the functions of HEK-Blue hTLR9/hMSR-1 cells

Figure 14 shows the results of the PAGE analysis of ssCpG and tetraCpG prepared at a DNA concentration of 100 μ M. ssCpG and tetraCpG are represented by single PAGE bands, indicating that tetraCpG had been prepared with high efficiency.

Figure 15 shows the MFI of the HEK-Blue hTLR9 cells, the mock-transfected HEK-Blue hTLR9 cells, and the HEK-Blue hTLR9/hMSR1 cells after the addition of Alexa Fluor 488-labeled DNA samples. The MFI values of the HEK-Blue hTLR9/hMSR1 cells were significantly higher than those of the mock-transfected HEK-Blue hTLR9 cells after addition of Alexa Fluor 488-ssCpG or tetraCpG. There was no significant difference in the MFI value of the HEK-Blue hTLR9/hMSR1 cells between Alexa Fluor 488-ssCpG and tetraCpG. In contrast, the MFI values of the cells after addition of Alexa Fluor 488-PS CpG2006 were not significantly different among the cells, irrespective of hMSR1 expression (data not shown).

Figure 16 shows SEAP activity after the addition of PS CpG2006, ssCpG, and tetraCpG. The HEK-Blue hTLR9 cells or mock-transfected HEK-Blue hTLR9 cells released SEAP upon the addition of PS CpG2006, but

did not release SEAP after the addition of ssCpG or tetraCpG. However, PS CpG2006, ssCpG, and tetraCpG all induced SEAP release from the HEK-Blue hTLR9/hMSR1 cells. There was no significant difference in SEAP activity after the addition of PS CpG2006 among the three types of cells. The SEAP activities of the HEK-Blue hTLR9/hMSR1 cells were comparable in both ssCpG and tetraCpG treatments.

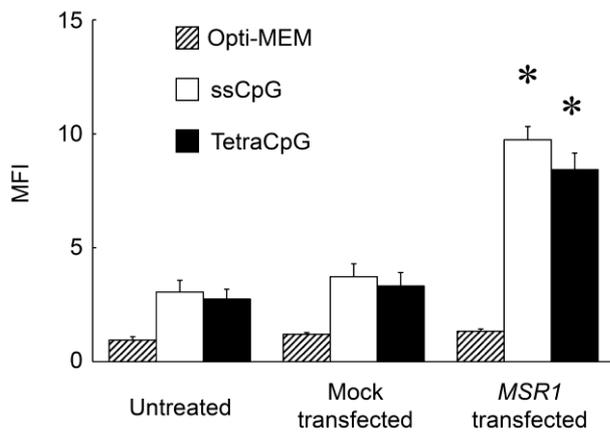


Figure 15. Uptake of ssCpG and tetraCpG in untreated, mock-transfected, or *MSR1*-transfected HEK-Blue hTLR9 cells. Each Alexa Fluor 488-labeled DNA sample was added to cells at a concentration of 2 $\mu\text{g}/\text{mL}$. The results are expressed as means + SEM of three independent experiments. * $P < 0.05$ compared with the mock-transfected group.

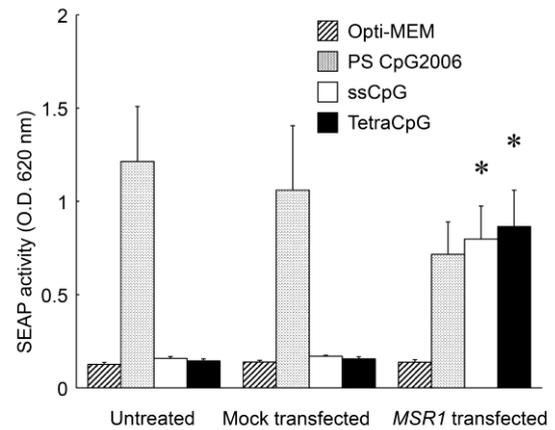


Figure 16. Secreted embryonic alkaline phosphatase (SEAP) release from untreated, mock-transfected, or *MSR1*-transfected HEK-Blue hTLR9 cells using HEK-Blue detection solution. Each DNA sample was added to the cells at a final concentration of 50 $\mu\text{g}/\text{mL}$, and the OD of the sample was measured at 620 nm. All oligodeoxynucleotides (ODNs) have a phosphodiester backbone except for CpG2006. The results are expressed as means + SEM of three independent experiments. * $P < 0.05$ compared with the mock-transfected group.

The uptake of Alexa Fluor 488-ssCpG and tetraCpG was examined in the presence of anti-hMSR1 antibody to confirm the involvement of hMSR1 in the uptake of DNA by HEK-Blue hTLR9/hMSR1 cells. Figure 17 shows the MFI of mock-transfected HEK-Blue hTLR9 cells and HEK-Blue hTLR9/hMSR1 cells after the addition of Alexa Fluor 488-labeled DNA samples in the presence of hMSR1 antibody or murine IgG1 isotype control antibody. Anti-hMSR1 antibody significantly reduced the uptake of Alexa Fluor 488-ssCpG and tetraCpG in the HEK-Blue hTLR9/hMSR1 cells.

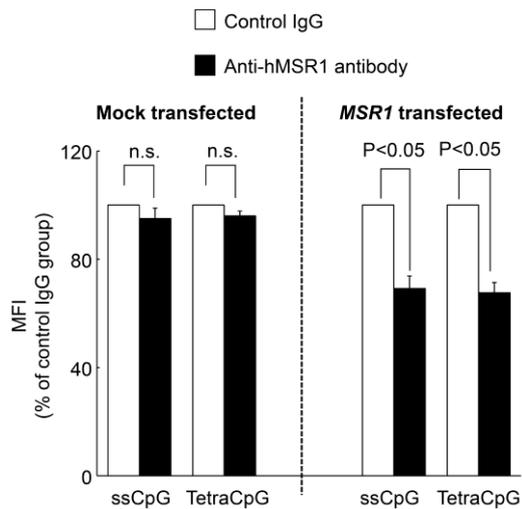


Figure 17. Cellular uptake of ssCpG and tetraCpG in mock-transfected or *MSR1*-transfected HEK-Blue hTLR9 cells in the presence of mouse IgG1 isotype control or hMSR1 antibody. Each Alexa Fluor 488-labeled DNA sample was added to cells at a final concentration of 2 $\mu\text{g}/\text{mL}$. The results are expressed as means + SEM of four independent experiments.

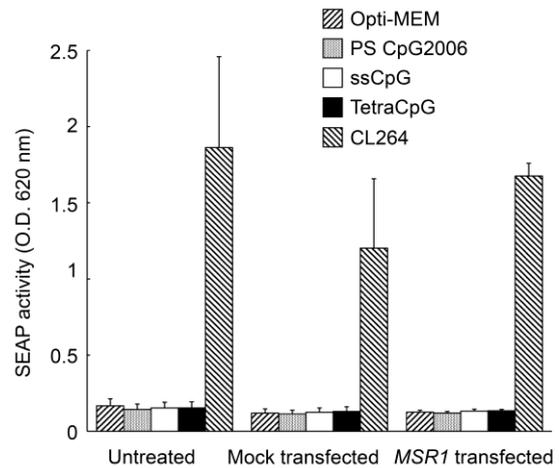


Figure 18. Secreted embryonic alkaline phosphatase (SEAP) release from untreated, mock-transfected, or *MSR1*-transfected HEK-Blue hTLR7 cells using a HEK-Blue detection solution. Each DNA sample was added to the cells at a final concentration of 50 $\mu\text{g}/\text{mL}$. The sample optical density (OD) was measured at 620 nm. The results are expressed as means + SEM of three independent experiments. CL264; a TLR7 ligand.

III-3-c. Experiments using HEK-Blue hTLR7 cells

To exclude the possibility that the expression of hMSR1 results in SEAP release without recognition of CpG DNA by TLR9, HEK-Blue hTLR7 cells were used instead of HEK-Blue hTLR9. The expression of hMSR1 in HEK-Blue hTLR7/hMSR1 cells was confirmed by western blotting (data not shown). Figure 18 shows SEAP activity after the addition of PS CpG2006, ssCpG, and tetraCpG to HEK-Blue hTLR7 cells. CL264, a TLR7 ligand, induced significant SEAP release. In contrast, PS CpG2006, ssCpG, and tetraCpG scarcely induced SEAP release from the HEK-Blue hTLR7 cells, irrespective of the expression of hMSR1. These results indicate that SEAP is released from HEK-Blue hTLR9/hMSR1 cells through the recognition of CpG DNA by TLR9.

III-4 Discussion

In the present study, I demonstrated that HEK-Blue hTLR9 cells efficiently responded to PS CpG DNA, which has high binding affinity for cell membranes, whereas they hardly responded to natural, PO CpG DNA, in spite of the fact that the cells expressed human TLR9. I also found that the low cellular uptake of PO CpG DNA by HEK-Blue hTLR9 cells explains their limited response to PO CpG DNA, and that transformation of the cells with a plasmid expressing hMSR1, a DNA receptor, restored the response of the cells to PO CpG DNA. Therefore, HEK-Blue hTLR9/hMSR1 cells can be used as a sensitive screening system for compounds that activate TLR9, although it could be difficult using HEK-Blue hTLR9/hMSR1 cells to discuss the physiological processes of the interaction of CpG DNA with TLR9-expressing cells or the mechanistic details of the cellular uptake of DNA.

The results of the present study suggest that hMSR1 or other DNA receptors are expressed in HEK-Blue hTLR9 or HEK293 cells. HEK293 cells, the parental cell line of HEK-Blue hTLR9 cells, have been widely used for the transfection of genes because they facilitate easy transformation. Therefore, HEK-Blue hTLR9 cells are also suitable for transfection studies. Both HEK293 cells and HEK-Blue hTLR9 cells hardly take up PO DNA, so they can be used to explore the receptors responsible for DNA binding, especially PO DNA binding.

MSR1 is a membrane protein that is located on the cell surface of macrophages and dendritic cells^{74,75}. I found that the hMSR1 expressed in HEK-Blue hTLR9/hMSR1 cells also localized at the cell membrane (Figure 13B). The MSR1 cDNA used in the present study contained hMSR1 signal-anchor sequences, so it is reasonable to assume that hMSR1 is appropriately sorted to the correct destination (the cell membrane). It has been reported that ligation to MSR1 induces clathrin-mediated endocytosis, and that the ligands are then sorted to endosomes⁷⁶. The mechanistic details of the uptake of DNA by HEK-Blue hTLR9/hMSR1 cells were not investigated in this study, but the efficient response to PO CpG DNA strongly suggests that the cells take up DNA in a similar manner to that adopted by other types of cells that express MSR1, such as dendritic cells.

Several reports suggest that hMSR1 is involved in the cellular uptake of PS CpG DNA⁷⁷. However, the present study demonstrated that hMSR-1 expression had no significant effect on the cellular uptake of Alexa Fluor 488-PS CpG DNA (data not shown) or on SEAP release (Figure 16). PS CpG DNA binds strongly to cell surfaces⁷⁸, and would mask any hMSR1-mediated cellular uptake of PS CpG DNA, even if it occurred.

Although most TLR9 is found in the endosomes, TLR9 is also detected on the surface of cells in some cell types^{79,80}. Some reports discussed that the cell surface TLR9 promoted the cellular uptake of CpG DNA as well as CpG DNA-coupled siRNA⁸¹⁻⁸³. In these studies, PS CpG DNA and the antisense strand of siRNA were conjugated and, therefore, strong binding of PS CpG DNA to the cell surface could lead to efficient uptake of the conjugate. Zhang et al. demonstrated that the cell surface TLR9 did not participate in the uptake of CpG DNA⁸². Therefore, the cell surface TLR9 on HEK-Blue hTLR9/hMSR1 cells, even if it exists, would not be critical for the immune responses to PO CpG DNA.

Previous studies from my laboratory demonstrated that in RAW264.7, DC2.4, and bone marrow-derived dendritic cells (BMDCs) nanostructured DNAs were taken up more efficiently than single-stranded or double-stranded DNAs^{24,25,38,73,66}. In the present study, I showed that hMSR1 can recognize nanostructured DNAs, such as tetrapodna. However, no significant differences were observed in the uptake by HEK-Blue hTLR9/hMSR1 cells between Alexa Fluor 488-ssCpG and Alexa Fluor 488-tetraCpG. These differences suggest that cell surface receptors other than MSR1 or auxiliary molecules are involved in the efficient cellular uptake of DNAs with complicated structures.

Taken together, the results of the present chapter demonstrate that the reconstruction of toll-like receptor 9-mediated responses to CpG DNA in HEK-Blue hTLR9 cells is useful for evaluating and predicting the TLR9-dependent toxicity of nucleic acid drug candidates, irrespective of their physicochemical properties. My results also suggest that the combination of HEK-Blue hTLR9 cells and natural PO CpG DNA can be used to

screen DNA receptors or DNA-binding proteins on the cell surface. Additional studies on other cell surface DNA receptors will improve understanding of the mechanisms underlying the interactions between DNA and cells at the molecular level⁸⁴.

Summary

Recently, DNA nanotechnology has attracted attention because of their ease of design. Many researchers including my laboratory reported that nanostructured DNA could be used for drug loading. However, there are little information about their delivery strategies, and is no report about the detail of the interaction of nanostructured DNA with immune cells. Nucleic acid drugs including CpG DNA need to be efficiently delivered to immune cells.

In this thesis, to clarify the characteristics of nanostructured DNA for efficient uptake of nucleic acid drugs by immune cells, I designed three big chapters.

In Chapter I, I aimed to elucidate the structural properties of nanostructured DNA to interact with immune cells. My laboratory previously reported that polypod-like structured DNA is useful for delivery of CpG DNA to immune cells such as macrophages or dendritic cells. Other group also reported that tetrahedral DNA could improve immune response from immune cells by CpG DNA. To clarify the structural property for efficient delivery, I elucidated immune response and physical properties using tetrapodna, tetrahedron, and tetragon. Then, a correlation was obtained between the structural properties and cellular uptake of nanostructured DNA. Moreover, I also revealed that nanostructured DNAs were more efficiently taken up by immune cells than by non-immune cells.

In Chapter II, I focused on structural rigidity and structural compactness of nanostructured DNA. To examine the effect of structural flexibility of DNA on their interaction with immune cells, I used DNA origami technology. Then, rectangular DNAs with many staples, more rigid structure, were efficiently interaction with RAW264.7 cells. Also, I elucidated using rolling circular amplification. More compact structure using cholesterol modified DNA was efficiently taken up by RAW264.7 cells.

In Chapter III, I tried to identify the cell surface receptor of nanostructured DNA. There are several reports about cell surface receptors of DNA, however their contribution to uptake of nanostructured DNA has not been fully elucidated. I elucidated their receptors transfected to HEK-Blue hTLR9 cells. I obtained the finding that scavenger receptor MSR1 is the cell surface receptor that recognizes nanostructured DNA.

In conclusion of my thesis, structural rigidity of nanostructured DNA is a key for their interaction with immune cells through cell surface receptors including MSR1.

Acknowledgements

I would like to express my sincere gratitude and thanks to Dr. Yoshinobu Takakura, Professor of Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan for giving me wonderful opportunities and their support with his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

I am extremely grateful to my research guides, Dr. Makiya Nishikawa, Professor of Laboratory of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Japan. It was a great opportunity to do my doctoral program under his guidance and to learn from his research expertise in Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Japan. All of his encouragements, insightful comments, and hard questions are fully helpful and essential for my Ph.D. course.

I wish to express my deepest appreciation to Dr. Yuki Takahashi, Associate Professor of Department of Biopharmaceutics and Drug Metabolism, Kyoto University, Japan for his patient supervision, insightful comments, suggestions, guidance and direction, valuable discussions and supports facilitating the successful completion of this study throughout the whole this study.

I highly appreciate the supports received through the collaborative work provided from Dr. Hiroshi Sugiyama, Professor of Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Japan, and Department of Chemistry, Graduate School of Science, Kyoto University, Japan; Dr. Masayuki Endo, Associate Professor of Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Japan. They kindly supported on data collection by Atomic force microscopy in Chapter I and Chapter II.

I also express my sincere appreciations to collaborative works provided from Dr. Norimitsu Kadowaki, Professor of Department of Internal Medicine, Faculty of Medicine, Kagawa University, Japan and Dr. Kenichi Ishiyama. They worked altogether with me in isolation of human PBMCs in Chapter I.

I would like to express my appreciations to collaborative works from Dr. Shinji Sakuma, Professor of Laboratory of Drug Delivery System, Faculty of Pharmaceutical Sciences, Setsunan University, Japan. He worked altogether with me in Section 3 of Chapter I.

I appreciate profoundly to Dr. Takao Inoue, Division of Molecular Target and Gene Therapy Products, National Institute of Health Sciences, Japan for his kind gift of HEK-Blue TLR cells and helpful guidance and discussions in Chapter III.

I also express my sincere appreciations to collaborative works provided from Dr. Mireille Lahoud, Associate Professor of Department of Biochemistry and Molecular Biology, Monash University, Australia. DEC-205 plasmid was kindly provided by her.

I would like to express sincere gratitude to Dr. Kohta Mohri, Mr. Noriyuki Matsuzaki, Ms. Yukako Shiba, Ms. Kanako Sugimura, Mr. Keisuke Umemura, Mr. Tatsuoki Maezawa, and Ms. Mutsumi Kariya for their great achievement and contribution as a co-author for my thesis, and their advice and warm support for this study.

I am greatly indebted to all members of Department of Biopharmaceutics and Drug metabolism and of Drug Delivery Research, Graduate School of Pharmaceutics Sciences, Kyoto University for their experimental assistance.

Finally, I would like to express my deepest gratitude to all of my family for their support, encouragements, and understanding throughout the doctoral course of this study.

List of Publications

Optimal arrangement of four short DNA strands for delivery of immunostimulatory nucleic acids to immune cells.

Shozo Ohtsuki, Noriyuki Matsuzaki, Kohta Mohri, Masayuki Endo, Tomoko Emura, Kumi Hidaka, Hiroshi Sugiyama, Yuki Takahashi, Kenichi Ishiyama, Norimitsu Kadowaki, Yoshinobu Takakura, Makiya Nishikawa.

Nucleic Acid Therapeutics **25**, 245-253 (2015).

Effect of the structural properties of tripod-like structured DNA on their interaction with macrophage-like RAW264.7 cells.

Shozo Ohtsuki, Yuki Takahashi, Yoshinobu Takakura, Makiya Nishikawa.

Manuscript in preparation

Elucidation of the mechanism of increased activity of immunostimulatory DNA by the formation of polypod-like structure.

Kohta Mohri, Kengo Nagata, Shozo Ohtsuki, Shiori Toyama, Mao Nonomura, Yuki Takahashi, Yoshinobu Takakura, Makiya Nishikawa, Shinji Sakuma.

Pharmaceutical Research **34**, 2362-2370 (2017).

Folding of single-stranded circular DNA into rigid rectangular DNA origami accelerates its cellular uptake.

Shozo Ohtsuki, Yukako Shiba, Tatsuoki Maezawa, Yuki Takahashi, Masayuki Endo, Tomoko Emura, Kumi Hidaka, Hiroshi Sugiyama, Yoshinobu Takakura, Makiya Nishikawa.

Manuscript in preparation

Development of DNA supramolecules formed by hydrophobic interaction for the effective delivery of CpG DNA.

Shozo Ohtsuki, Kanako Sugimura, Yuki Takahashi, Mutsumi Kariya, Makiya Nishikawa, Yoshinobu Takakura.

Manuscript in preparation

Reconstruction of Toll-like receptor 9-mediated response to CpG DNA in HEK-Blue hTLR9 cells by transfection of human macrophage scavenger receptor 1 gene.

Shozo Ohtsuki, Yuki Takahashi, Takao Inoue, Yoshinobu Takakura, Makiya Nishikawa.

Scientific Reports **7**, 13661 (2017).

Other Publications

Self-assembling DNA dendrimer for effective delivery of immunostimulatory CpG DNA to immune cells.

Kohta Mohri, Eri Kusuki, Shozo Ohtsuki, Natsuki Takahashi, Masayuki Endo, Kumi Hidaka, Hiroshi Sugiyama, Yuki Takahashi, Yoshinobu Takakura, Makiya Nishikawa.

Biomacromolecules **16**, 1095-1101 (2015).

Self-assembling DNA hydrogel-based delivery of immunoinhibitory nucleic acids to immune cells.

Yu Nishida, Shozo Ohtsuki, Yuki Araie, Yuka Umeki, Masayuki Endo, Tomoko Emura, Kumi Hidaka, Hiroshi Sugiyama, Yuki Takahashi, Yoshinobu Takakura, Makiya Nishikawa.

Nanomedicine **12**, 123-130 (2016).

DNA nanotechnology-based composite-type gold nanoparticle-immunostimulatory DNA hydrogel for tumor photothermal immunotherapy.

Tomoya Yata, Yuki Takahashi, Mengmeng Tan, Hiroataka Nakatsuji, Shozo Ohtsuki, Tatsuya Murakami, Hiroshi Imahori, Yuka Umeki, Tomoki Shiomi, Yoshinobu Takakura, Makiya Nishikawa.

Biomaterials **146**, 136-145 (2017).

References

1. Fattal, E. and Bochot, A. Ocular delivery of nucleic acids: antisense oligonucleotides, aptamers and siRNA. *Adv. Drug Deliv. Rev.* **58**, 1203–1223 (2006).
2. Dove, A. Antisense and sensibility. *Nat. Biotechnol.* **20**, 121–124 (2002).
3. Chen, C., Yang, Z. and Tang, X. Chemical modifications of nucleic acid drugs and their delivery systems for gene-based therapy. *Med. Res. Rev.* 1–41 (2018). doi:10.1002/med.21479
4. Khvorova, A. and Watts, J. K. The chemical evolution of oligonucleotide therapies of clinical utility. *Nat. Biotechnol.* **35**, 238–248 (2017).
5. Kanasty, R., Dorkin, J. R., Vegas, A. and Anderson, D. Delivery materials for siRNA therapeutics. *Nat. Mater.* **12**, 967–977 (2013).
6. Khorkova, O. and Wahlestedt, C. Oligonucleotide therapies for disorders of the nervous system. *Nat. Biotechnol.* **35**, 249–263 (2017).
7. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. A Toll-like receptor recognizes bacterial DNA A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745 (2000).
8. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J. and Krieg, A. M. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2879–83 (1996).
9. Krug, A., Rothenfusser, S., Hornung, V., Jahrsdrfer, B., Blackwell, S., Ballas, Z. K., Endres, S., Krieg, A. M. and Hartmann, G. Identification of CpG oligonucleotide sequences with high induction of IFN- α/β in plasmacytoid dendritic cells. *Eur. J. Immunol.* **31**, 2154–2163 (2001).
10. Wagner, H. Toll meets bacterial CpG-DNA. *Immunity* **14**, 499–502 (2001).
11. Krieg, A. M. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol.* **20**, 709–760 (2002).
12. Klinman, D. M. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **4**, 249–259 (2004).
13. Krieg, A. M. Therapeutic potential of toll-like receptor 9 activation. *Nat. Rev. Drug Discov.* **5**, 471–484 (2006).
14. Vollmer, J. and Krieg, A. M. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv. Drug Deliv. Rev.* **61**, 195–204 (2009).
15. Kawai, T. and Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity* **34**, 637–650 (2011).
16. Gupta, G. K. and Agrawal, D. K. CpG Oligodeoxynucleotides as TLR9 Agonists. **24**, 225–235 (2010).
17. Rothmund, P. W. K. Folding DNA to create nanoscale shapes and patterns. *Nature* **440**, 297–302 (2006).
18. Zhang, C., Su, M., He, Y., Zhao, X., Fang, P., Ribbe, A. E., Jiang, W. and Mao, C. Conformational flexibility facilitates self-assembly of complex DNA nanostructures. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 10665–10669

(2008).

19. He, Y., Ye, T., Su, M., Zhang, C., Ribbe, A. E., Jiang, W. and Mao, C. Hierarchical self-assembly of DNA into symmetric supramolecular polyhedra. *Nature* **452**, 198–201 (2008).
20. Um, S. H., Lee, J. B., Park, N., Kwon, S. Y., Umbach, C. C. and Luo, D. Enzyme-catalysed assembly of DNA hydrogel. *Nat. Mater.* **5**, 797–801 (2006).
21. Schmidt, T. L. and Heckel, A. Construction of a structurally defined double-stranded DNA catenane. *Nano Lett.* **11**, 1739–1742 (2011).
22. Nishikawa, M., Matono, M., Rattanakiat, S., Matsuoka, N. and Takakura, Y. Enhanced immunostimulatory activity of oligodeoxynucleotides by Y-shape formation. *Immunology* **124**, 247–255 (2008).
23. Nishikawa, M., Mizuno, Y., Mohri, K., Matsuoka, N., Rattanakiat, S., Takahashi, Y., Funabashi, H., Luo, D. and Takakura, Y. Biodegradable CpG DNA hydrogels for sustained delivery of doxorubicin and immunostimulatory signals in tumor-bearing mice. *Biomaterials* **32**, 488–494 (2011).
24. Mohri, K., Nishikawa, M., Takahashi, N., Shiomi, T., Matsuoka, N., Ogawa, K., Endo, M., Hidaka, K., Sugiyama, H., Takahashi, Y. and Takakura, Y. Design and development of nanosized DNA assemblies in polypod-like structures as efficient vehicles for immunostimulatory cpg motifs to immune cells. *ACS Nano* **6**, 5931–5940 (2012).
25. Uno, S., Nishikawa, M., Mohri, K., Umeki, Y., Matsuzaki, N., Takahashi, Y., Fujita, H., Kadowaki, N. and Takakura, Y. Efficient delivery of immunostimulatory DNA to mouse and human immune cells through the construction of polypod-like structured DNA. *Nanomedicine Nanotechnology, Biol. Med.* **10**, 765–774 (2014).
26. Li, J., Fan, C., Pei, H., Shi, J. and Huang, Q. Smart drug delivery nanocarriers with self-assembled DNA nanostructures. *Adv. Mater.* **25**, 4386–4396 (2013).
27. Nishikawa, M., Ogawa, K., Umeki, Y., Mohri, K., Kawasaki, Y., Watanabe, H., Takahashi, N., Kusuki, E., Takahashi, R., Takahashi, Y. and Takakura, Y. Injectable, self-gelling, biodegradable, and immunomodulatory DNA hydrogel for antigen delivery. *J. Control. Release* **180**, 25–32 (2014).
28. Nishikawa, M., Rattanakiat, S. and Takakura, Y. DNA-based nano-sized systems for pharmaceutical and biomedical applications. *Adv. Drug Deliv. Rev.* **62**, 626–632 (2010).
29. Matsuoka, N., Nishikawa, M., Mohri, K., Rattanakiat, S. and Takakura, Y. Structural and immunostimulatory properties of Y-shaped DNA consisting of phosphodiester and phosphorothioate oligodeoxynucleotides. *J. Control. Release* **148**, 311–316 (2010).
30. Hancock, R. E. W., Nijnik, A. and Philpott, D. J. Modulating immunity as a therapy for bacterial infections. *Nat. Rev. Microbiol.* **10**, 243–254 (2012).
31. O’Neill, L. A. J., Hennessy, E. J. and Parker, A. E. Targeting Toll-like receptors: Emerging therapeutics? *Nat. Rev. Drug Discov.* **9**, 293–307 (2010).
32. Li, J., Pei, H., Zhu, B., Liang, L., Wei, M., He, Y., Chen, N., Li, D., Huang, Q. and Fan, C. Self-assembled multivalent DNA nanostructures for noninvasive intracellular delivery of immunostimulatory CpG

- oligonucleotides. *ACS Nano* **5**, 8783–8789 (2011).
33. Kawamura, K., Kadowaki, N., Kitawaki, T. and Uchiyama, T. Virus-stimulated plasmacytoid dendritic cells induce CD4⁺ cytotoxic regulatory T cells. *Control* **107**, 1031–1038 (2006).
 34. Endo, M., Katsuda, Y., Hidaka, K. and Sugiyama, H. Regulation of DNA methylation using different tensions of double strands constructed in a defined DNA nanostructure. *J. Am. Chem. Soc.* **132**, 1592–1597 (2010).
 35. Benimetskaya, L., Loike, J. D., Khaled, Z., Loike, G., Silverstein, S. C., Cao, L., El Khoury, J., Cai, T. Q. and Stein, C. A. Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nat. Med.* **3**, 414–420 (1997).
 36. Siess, D. C., Vedder, C. T., Merckens, L. S., Tanaka, T., Freed, A. C., McCoy, S. L., Heinrich, M. C., Deffebach, M. E., Bennett, R. M. and Hefeneider, S. H. A human gene coding for a membrane-associated nucleic acid-binding protein. *J. Biol. Chem.* **275**, 33655–33662 (2000).
 37. Tversky, J. R., Le, T. V., Bieneman, A. P., Chichester, K. L., Hamilton, R. G. and Schroeder, J. T. Human blood dendritic cells from allergic subjects have impaired capacity to produce interferon- α via toll-like receptor 9. *Clin. Exp. Allergy* **38**, 781–788 (2008).
 38. Ohtsuki, S., Matsuzaki, N., Mohri, K., Endo, M., Emura, T., Hidaka, K., Sugiyama, H., Takahashi, Y., Ishiyama, K., Kadowaki, N., Takakura, Y. and Nishikawa, M. Optimal Arrangement of Four Short DNA Strands for Delivery of Immunostimulatory Nucleic Acids to Immune Cells. *Nucleic Acid Ther.* **25**, 245–53 (2015).
 39. Chithrani, B. D., Ghazani, A. A. and Chan, W. C. W. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. *Nano Lett.* **6**, 662–668 (2006).
 40. He, C., Hu, Y., Yin, L., Tang, C. and Yin, C. Effects of particle size and surface charge on cellular uptake and biodistribution of polymeric nanoparticles. *Biomaterials* **31**, 3657–3666 (2010).
 41. Yin Win, K. and Feng, S. S. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. *Biomaterials* **26**, 2713–2722 (2005).
 42. Zhong, M. and Kallenbach, N. R. Conformation and Thermodynamics of DNA ‘Necks’; Models for Three-arm Branch Formation in a Duplex. *Journal of molecular biology* **230**, 766–778 (1993).
 43. Swanson, J. a and Watts, C. Macropinocytosis. *Trends Cell Biol.* **5**, 424–428 (1995).
 44. Swanson, J. A. Shaping cups into phagosomes and macropinosomes. *Nat. Rev. Mol. Cell Biol.* **9**, 639–649 (2008).
 45. Conner, S. D. and Schmid, S. L. Regulated portals of entry into the cell. *Nature* **422**, 37–44 (2003).
 46. Doherty, G. J. and McMahon, H. T. Mechanisms of Endocytosis. *Annu. Rev. Biochem.* **78**, 857–902 (2009).
 47. Oh, N. and Park, J. H. Endocytosis and exocytosis of nanoparticles in mammalian cells. *Int. J. Nanomedicine* **9**, 51–63 (2014).
 48. Liedl, T., Högberg, B., Tytell, J., Ingber, D. E. and Shih, W. M. Self-assembly of three-dimensional prestressed tensegrity structures from DNA. *Nat. Nanotechnol.* **5**, 520–524 (2010).
 49. Andersen, E. S., Dong, M., Nielsen, M. M., Jahn, K., Subramani, R., Mamdouh, W., Golas, M. M., Sander, B., Stark, H., Oliveira, C. L. P., Pedersen, J. S., Birkedal, V., Besenbacher, F., Gothelf, K. V. and Kjems, J.

- Self-assembly of a nanoscale DNA box with a controllable lid. *Nature* **459**, 73–76 (2009).
50. Subramanian, H. K. K., Chakraborty, B., Sha, R. and Seeman, N. C. The Label-Free Unambiguous Detection and Symbolic Display of Single Nucleotide Polymorphisms on DNA Origami. 910–913 (2011).
doi:10.1021/nl104555t
 51. Movshovich, R., Vekhter, I., Pagliuso, P. G., Sarrao, J. L., Paglione, J., Taillefer, L., Ovchinnikov, Y. N., Ferrell, R. A., Shimahara, H., Movshovich, R., Capan, C., Pagliuso, P. G., Sarrao, J. L., Dewhurst, C. D., Hoogenboom, B. W., Canfield, P. C., Dewhurst, C. D., Hoogenboom, B. W., Eskildsen, M. R., *et al.* Self-Assembled Water-Soluble Nucleic Acid Probe Tiles for Label-Free RNA Hybridization Assays. 180–184 (2008).
 52. Fire, A. and Xu, S. Q. Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci.* **92**, 4641–4645 (1995).
 53. Liu, D., Daubendiek, S. L., Zillman, M. A., Ryan, K. and Kool, E. T. Rolling circle DNA synthesis: Small circular oligonucleotides as efficient templates for DNA polymerases. *J Am Chem Soc* **118**, 1587–1594 (1996).
 54. Cavagnaro Joy, Berman Cindy, Kornbrust Doug, White Tacey, Campion Sarah, and H. S. Considerations for Assessment of Reproductive and Developmental Toxicity of Oligonucleotide-Based Therapeutics. *Nucleic Acid Ther.* **24**, 313–325 (2014).
 55. Kleinman, M. E., Yamada, K., Takeda, A., Chandrasekaran, V., Nozaki, M., Baffi, J. Z., Albuquerque, R. J. C., Yamasaki, S., Itaya, M., Pan, Y., Appukuttan, B., Gibbs, D., Yang, Z., Karikó, K., Ambati, B. K., Wilgus, T. A., DiPietro, L. A., Sakurai, E., Zhang, K., *et al.* Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* **452**, 591–597 (2008).
 56. Kariko, K., Bhuyan, P., Capodici, J. and Weissman, D. Small Interfering RNAs Mediate Sequence-Independent Gene Suppression and Induce Immune Activation by Signaling through Toll-Like Receptor 3. *J. Immunol.* **172**, 6545–6549 (2004).
 57. Hornung, V., Guenther-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., De Fougerolles, A., Endres, S. and Hartmann, G. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* **11**, 263–270 (2005).
 58. Judge, A. D., Sood, V., Shaw, J. R., Fang, D., McClintock, K. and MacLachlan, I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* **23**, 457–462 (2005).
 59. Marjorie Robbins, Adam Judge, and I. M. siRNA and Innate Immunity. *Oligonucleotides* **19**, 89–102 (2009).
 60. Sioud, M. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J. Mol. Biol.* **348**, 1079–1090 (2005).
 61. Sledz, C. A., Holko, M., De Veer, M. J., Silverman, R. H. and Williams, B. R. G. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**, 834–839 (2003).
 62. Krieg, A. M. CpG motifs: The active ingredient in bacterial extracts? *Nat. Med.* **9**, 831–835 (2003).

63. Krug, A., French, A. R., Barchet, W., Fischer, J. A. A., Dzionek, A., Pingel, J. T., Orihuela, M. M., Akira, S., Yokoyama, W. M. and Colonna, M. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* **21**, 107–119 (2004).
64. Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T. and Golenbock, D. T. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* **5**, 190–198 (2004).
65. Rutz, M., Metzger, J., Gellert, T., Lippa, P., Lipford, G. B., Wagner, H. and Bauer, S. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur. J. Immunol.* **34**, 2541–2550 (2004).
66. Mohri, K., Kusuki, E., Ohtsuki, S., Takahashi, N., Endo, M., Hidaka, K., Sugiyama, H., Takahashi, Y., Takakura, Y. and Nishikawa, M. Self-Assembling DNA Dendrimer for Effective Delivery of Immunostimulatory CpG DNA to Immune Cells. *Biomacromolecules* **16**, 1095–1101 (2015).
67. Kimura, Y., Sonehara, K., Kuramoto, E., Makino, T., Yamamoto, S., Yamamoto, T., Kataoka, T. and Tokunaga, T. Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. *J. Biochem.* **116**, 991–994 (1994).
68. Sirois, C. M., Jin, T., Miller, A. L., Bertheloot, D., Nakamura, H., Horvath, G. L., Mian, A., Jiang, J., Schrum, J., Bossaller, L., Pelka, K., Garbi, N., Brewah, Y., Tian, J., Chang, C., Chowdhury, P. S., Sims, G. P., Kolbeck, R., Coyle, A. J., *et al.* RAGE is a nucleic acid receptor that promotes inflammatory responses to DNA. *J. Exp. Med.* **210**, 2447–2463 (2013).
69. Moseman, A. P., Moseman, E. A., Schworer, S., Smirnova, I., Volkova, T., von Andrian, U. and Poltorak, A. Mannose Receptor 1 Mediates Cellular Uptake and Endosomal Delivery of CpG-Motif Containing Oligodeoxynucleotides. *J. Immunol.* **191**, 5615–5624 (2013).
70. Caminschi, I., Meuter, S. and Heath, W. R. DEC-205 is a cell surface receptor for CpG oligonucleotides. *Oncoimmunology* **2**, (2013).
71. Nakano, T. & Yamamura, E. Patent WO2014088087 A1; PCT/JP2013/082774. (2014).
72. Haile, L. A., Puig, M., Kelley-Baker, L. and Verthelyi, D. Detection of innate immune response modulating impurities in therapeutic proteins. *PLoS One* **10**, (2015).
73. Nishida, Y., Ohtsuki, S., Araie, Y., Umeki, Y., Endo, M., Emura, T., Hidaka, K., Sugiyama, H., Takahashi, Y., Takakura, Y. and Nishikawa, M. Self-assembling DNA hydrogel-based delivery of immunoinhibitory nucleic acids to immune cells. *Nanomedicine Nanotechnology, Biol. Med.* **12**, 123–130 (2016).
74. Platt, N. and Gordon, S. Scavenger receptors: diverse activities and promiscuous binding of polyanionic ligands. *Chem. Biol.* **5**, R193-203 (1998).
75. Platt, N. and Gordon, S. Is the class A macrophage scavenger receptor (SR-A) multifunctional? — The mouse 's tale. *J. Clin. Investig.* **108**, 649–654 (2001).
76. Zani, I., Stephen, S., Mughal, N., Russell, D., Homer-Vanniasinkam, S., Wheatcroft, S. and Ponnambalam, S. Scavenger Receptor Structure and Function in Health and Disease. *Cells* **4**, 178–201 (2015).

77. Jozefowski, S. Role of scavenger receptor MARCO in macrophage responses to CpG oligodeoxynucleotides. *J. Leukoc. Biol.* **80**, 870–879 (2006).
78. Kurreck, J. Antisense technologies: Improvement through novel chemical modifications. *Eur. J. Biochem.* **270**, 1628–1644 (2003).
79. Eaton-Bassiri, A., Dillon, S. B., Cunningham, M., Ryczyn, M. A., Mills, J., Sarisky, R. T. and Mbow, M. L. Toll-like receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells. *Infect Immun* **72**, 7202–7211 (2004).
80. Saikh, K. U., Kissner, T. L., Sultana, A., Ruthel, G. and Ulrich, R. G. Human Monocytes Infected with *Yersinia pestis* Express Cell Surface TLR9 and Differentiate into Dendritic Cells. *J. Immunol.* **173**, 7426–7434 (2004).
81. Nechaev, S., Gao, C., Moreira, D., Swiderski, P., Jozwiak, A., Kowolik, C. M., Zhou, J., Armstrong, B., Raubitschek, A., Rossi, J. J. and Kortylewski, M. Intracellular processing of immunostimulatory CpG-siRNA: Toll-like receptor 9 facilitates siRNA dicing and endosomal escape. *J. Control. Release* **170**, 307–315 (2013).
82. Zhang, Q., Hossain, D. S., Nechaev, S., Kozłowska, A., Zhang, W., Liu, Y., Kowolik, C. M., Swiderski, P., Rossi, J. J., Forman, S., Pal, S., Bhatia, R., Raubitschek, A., Yu, H. and Kortylewski, M. TLR9-mediated siRNA delivery for targeting of normal and malignant human hematopoietic cells in vivo. **121**, 1304–1315 (2013).
83. Kortylewski, M., Swiderski, P., Herrmann, A., Wang, L., Kowolik, C., Kujawski, M., Lee, H., Scuto, A., Liu, Y., Yang, C., Deng, J., Soifer, H. S., Raubitschek, A., Forman, S., Rossi, J. J., Pardoll, D. M., Jove, R. and Yu, H. In vivo delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat. Biotechnol.* **27**, 925–932 (2009).
84. Ohtsuki, S., Takahashi, Y., Inoue, T., Takakura, Y. and Nishikawa, M. Reconstruction of Toll-like receptor 9-mediated responses in HEK-Blue hTLR9 cells by transfection of human macrophage scavenger receptor 1 gene. *Sci. Rep.* **7**, 13661 (2017).