Modular delivery of CpG-incorporated lipid-DNA nanoparticles for spleen DC activation

Jun-O Jin, Haein Park, Wei Zhang, Jan Willem de Vries, Agnieszka Gruszka, Myung Won Lee, Dae-Ro Ahn, Andreas Herrmann, Minseok Kwak

Shanghai Public Health Clinical Center, Shanghai Medical College, Fudan University, Shanghai 201508, China
Department of Chemistry, Pukyong National University, 45 Yongso-ro, Nam-gu, Busan 48513, Republic of Korea
University of Groningen, Zernike Institute for Advanced Materials, Department of Polymer Chemistry, Nijenborgh 4, 9747 AG Groningen, The Netherlands
Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, 5 Hwarangno 14-gil, Seongbuk-gu, Seoul 02792, Republic of Korea

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ABSTRACT

We introduce a versatile carrier system for in vitro and in vivo immune stimulation based on soft matter DNA nanoparticles (NPs). The incorporation of lipid-modified nucleotides into DNA strands enables the formation of micelles of uniform size. In a single self-assembly step, the micelles can be equipped with immune adjuvant (CpG) motifs and fluorescent probes. The immunological effects of CpG confined at the NP surface were studied in a comprehensive manner in animal experiments. Dose-dependent activation of spleen dendritic cells (DCs) by CpG-conjugated NP was observed, which was accompanied by the pronounced up-regulation of co-stimulatory molecule and cytokine production.

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1. Introduction

For over 30 years, we have witnessed a rapid growth of DNA nanotechnology [1]. Since Seeman’s pioneering work, “DNA in a material world” is recognized as a promising scaffolding approach in various research disciplines [2]. The most basic principle in the field is the programmability of DNA sequences so that highly precise DNA nanostructures can be prepared based on specific motifs, such as DNA junctions and crossovers used in DNA polyhedra and DNA origamis, respectively [3–5]. The formation of such complex and beautiful structures requires the incorporation of multiple synthetic oligonucleotides (a few to several hundred) and/or biologically derived high molecular weight polynucleotides (e.g., plasmids of M13 bacteriophage for DNA origamis).

Another approach of constructing DNA nanostructures makes use of the partitioning of hydrophobic units in an aqueous environment. When hydrophobic units are covalently attached to DNA, such as polymers or lipids, oligonucleotide-based amphiphiles are obtained that undergo microphase separation to self-assemble into micelle structures [6]. It has been demonstrated that the shape and size of these objects can be manipulated by the actions of enzymes or simple and mild hybridization procedures resulting in shape-dependent cellular uptake [7,8]. Compared to pristine DNA nanoobjects, these structures are not held in place by Watson-Crick base pairing, but by hydrophobic interactions. The remaining single-stranded sequences can be functionalized by hybridization with complementary functionalized oligonucleotides. When decorated with a targeting ligand, such soft matter DNA nanoparticles have been employed for the delivery of anticancer drugs [9]. On the other hand, the hydrophobic segments of the DNA amphiphiles can be interfaced with other materials, such as carbon nanotubes, silicon nanowires, and vesicles [10–12]. This successful integration of these kind of DNA nanostructures into intricate systems is greatly facilitated by their low complexity and low number of constituent components, their high density of surface-exposed single-stranded oligonucleotides, and their facile and scalable (up to several hundreds of mg) fabrication procedures based on solid-phase synthesis or solution-phase chemistry [13]. These features also qualify this type of multifunctional nanomaterials for future biological and biomedical studies.

Dendritic cells (DCs) are antigen-presenting cells (APCs) and activators for T- and B-cells, mainly owing to their ability to take up
and present antigens (AGs) [14–16]. DCs exist in two functionally and phenotypically distinct stages as immature and mature DCs. Upon exposure to AGs, pathogen components, or microbial stimuli, DCs undergo maturation and present AGs to T cells [14,15,17]. This transition of DCs is characterized by the increased expression of co-stimulatory molecules, production of pro-inflammatory cytokines, and further presentation of AGs to T cells [18]. In particular, DCs can directly sense pathogen components by pattern recognizing receptors, such as toll like receptors (TLRs), scavenger receptors, and complement receptors [15,18–20]. Unmethylated cytosine-phosphate-guanosine oligodeoxyribonucleotides (CpG-ODNs), which are TLR-9 agonists, have been widely explored in DC activation and cancer immunotherapy, as CpG-stimulated DC potentially promotes T helper 1 (Th1) cells and cytotoxic T lymphocyte (CTL) activation [21–23]. DNA-based nanostructures have been used as delivery platforms for TLR agonists, such as CpG, due to their superb biocompatibility and straightforward incorporation of nucleic acids. The dimension and multivalence of the DNA-based carriers containing CpG ligands play crucial roles for TLR-mediated DC activation as shown for DNA tetrahedron [24] and tubular DNA origami [25]. However, these delivery vehicles of TLR agonists to DCs and their stimulatory effect on DCs have only been investigated in vitro using primary cell culture systems [13,23,26,27]. To develop such carriers further and to analyze their complex immune orchestration, evaluating DNA-based delivery platforms and immune activation in vivo is required, especially for the maturation of spleen DCs, as well as for further applications, such as immunotherapy and vaccination.

In this study, we hypothesized that spherical soft matter nucleic acid nanoparticles composed of DNA amphiphiles can readily be equipped with a number of immunostimulatory adjuvants (Fig. 1a) and may be cleared by endonucleases. In addition, thanks to simple and well-established synthetic protocols, substantial amounts of DNA nanomaterials can be administrated in vivo to perform a series of immune-stimulatory experiments. Consequently, we analyzed the full immunological assay, including phagocytosis, the expression of co-stimulatory molecules, and the production of pro-inflammatory cytokines in spleen DCs (Fig. 1b and c). We studied dose-dependent DC activation of lipid-DNA nanoparticles with different CpG coverages through a simple stoichiometric incorporation of the CpGs onto the surface of nanoparticles. The results from soft matter DNA hybrid nanoparticles were compared with a pristine DNA polyhedron-based CpG delivery system.

2. Results and discussion
2.1. Materials characterization
2.1.1. DNA materials
A 12-nucleotide (NT) lipid-DNA strand, which contains four consecutive dodec-1-ynyluracil nucleobases at the 5′-terminus, namely U4T (5′-UUUU UGC GGA TTC-3′; the underline denotes lipid-modified deoxyribouracil), was selected as the basic building block for our immunostimulating delivery system (Fig. 1a) [28]. The lipid-DNA was synthesized as reported previously [29] in 50–250 μmol to yield sufficient amounts (at least 40 mg–200 mg) required for in vivo experiments. As anticipated, the fast protein liquid chromatography (FPLC)-purified U4T (see Fig. S1 for a representative FPLC elugram of crude U4T) exhibited aggregation above its critical micelle concentration (1.3 μM) due to its amphiphilic structure. With the help of transmission electron microscopy (TEM), the size of spherical micelles composed of single-stranded (ss) U4T (20 μM) was revealed (diameter 8.1 ± 1.3 nm, Fig. S2a), which is in good agreement with analogous ss lipid-DNA nanoobjects measured by the dynamic light scattering technique (6.7–7.9 nm hydrodynamic diameter) [28]. To transform the DNA nanoparticles into immune-stimulating carriers, we decorated the surface with a particular CpG-ODN sequence. The ODN 1826 (5′-TCC ATG ACG TTC CTG ACG TT-3′) is the most frequently utilized TLR-9 specific ligand and is a representative immune-adjuvant. For functionalizing the rim of the DNA nanoparticles with this sequence, the 3′-end of the ODN 1826 (20 nt) was extended with the 12 nt long complement of U4T (CU4T: 5′-GA A GCC AAA 3′). The combination of both sequences allowed direct functionalization of the nanoparticle surface with the adjuvant through hybridization of U4T and eCpG (5′-ODN 1826-cU4T-3′; 5′-TCC ATG ACG TTC CCG TAT AGG CAA AA-3′). Upon hybridization of U4T and eCpG, the nanoobjects exhibited diameters of 7.9 ± 0.9 nm as determined from TEM images (Fig. S2b). The sizes of pristine U4T (ss) and double-stranded U4T decorated with eCpG (ds U4T-eCpG, later on called INP) did not differ notably. One expects a larger diameter for ds U4T-eCpG than for ss U4T; however, the ss segment containing the CpG motif protruding from the micelle might yield a low contrast under TEM imaging conditions and is therefore not detected (Fig. S2c).

2.1.2. Serum stability of INP
Additionally, we performed a stability assay for the immunostimulatory nanoparticles (INP) against nucleases because this material is intended to be administered to living animals. To determine the half-life of INP, fully covered with eCpG, we incubated a set of samples in test tubes for different time periods (0–12 h) with serum-containing buffer—non-inactivated fetal bovine serum (FBS, 10% v/v) in Dulbecco’s modified Eagle medium (DMEM) [30]. By electrophoretic analysis, a half-life of INP was determined to be approximately 30 min, which is 120 times longer than the one-pass blood circulation time (15 s) of a mouse (Fig. S3) [31]. These experiments suggest that our INP has a similar stability as ss- or dsDNA against exonucleases in serum due to the freely accessible DNA exposed on the INP surface [24,30].
2.2. DC experiment in vitro

2.2.1. Activation of BMDCs by INP

Prior to using INP as an immunostimulation agent, we examined whether INP is cytotoxic. The RAW264.7 cells were treated with CpG and different volumes of INP for 24 h and showed that all materials did not induce cytotoxicity in the RAW264.7 cells (Fig. S4).

Next, to determine whether INP can induce activation of bone marrow-derived DCs (BMDCs), we treated the cells with INPs. BMDCs from C57BL/6 mice were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 6 days. The cells were further treated with 1, 10, and 50 μL of INP, where 80 μM U4T was occupied by 40 μM eCpG, in phosphate buffer saline (PBS) for 24 h, using pristine CpG as a positive control. Treatment with INP at 10 and 50 μL dramatically promoted dendritic morphological changes in BMDCs (Fig. 2a) and markedly up-regulated the expression of the MHC class II and co-stimulatory molecules CD40, CD80 and CD86 (Fig. 2b and c).

Moreover, the levels of IL-6, IL-12p40, and TNF-α mRNA in INP-treated BMDCs were significantly increased compared to the PBS-treated BMDCs (Fig. 2d). Consistent with mRNA expression levels, the secretion levels of IL-6, IL-12p70 and TNF-α in cultured medium of BMDCs treated with INPs were dramatically increased compared to those treated with PBS (Fig. 2e). In addition, up-regulation of co-stimulatory molecules and pro-inflammatory cytokines induced by INP was considerably higher than that of the pristine CpG alone. Thus, with these in vitro results we concluded that INPs can potentially play a role to enhance activation of BMDCs than CpG not confined in an amphiphilic nanoparticle.

2.3. DC experiment in vivo

2.3.1. INP uptake to DC

To determine whether INP can be delivered to mouse spleen DC in vivo, we administered fluorescently labeled INP intravenously (i.v.) to C57BL/6 mice (the most widely used genetically modified mouse model for human disease and immunology studies) to allow for the detection of DCs that have phagocytosed the INP. For this uptake experiment, we simply hybridized ss U4T particles with one quarter of 5'-Alexa488-labeled cU4T (Alexa-cU4T) and one quarter of eCpG. Two hours after the injection, we harvested spleen and analyzed splenocytes by multi-color flow cytometry. It was found that Alexa488+ cells were substantially enriched in CD11c+ cells, whereas CD11c– cells did not increase (Fig. 3a). More than 40% of spleen DCs, defined as lineage CD11c+ cells (Fig. S5), were Alexa488 positive (Fig. 3b). To further confirm this observation, we stained CD11c and TLR9 in the spleen section of the mouse that received i.v. with INP-Alexa for 2 h. As presented in Fig. 3c, indeed, a large proportion of CD11c+ spleen DCs were stained by Alexa488’s characteristic green fluorescence. Moreover, the Alexa488 was co-localized intracellular TLR9 in the spleen CD11c+ DCs. In addition, pre-treatment of ODN 2088, a murine TLR9 antagonist, in the mice completely inhibited phagocytosis of INPs in the spleen DCs (Fig. 3d–e). This uptake experiment implies that TLR9 was constitutively expressed in the endoplasmic reticulum in the cytoplasm before immune activation and moved outward to the endolysosomes after being activated by its ligand, eCpG herein [32]. The fluorescence was mostly co-localized in cytoplasm TLR9 of the spleen DCs, which indicated that INP was successfully and efficiently delivered to the cytoplasm of spleen DCs. In addition, we also examined the phagocytosis of INPs into spleen DCs in CpG dosage-dependent manner. To vary CpG population extruded on the surface of soft matter nanoparticle, we prepared Alexa488-occupied (1q) INPs that possess different portions of eCpG: one, two, and three quarter of ss U4T was hybridized with eCpG, namely INP1q, 2q and 3q, respectively. Interestingly, there was no notable difference in phagocytosis of INPs with varied CpG concentrations (Fig. S6). This result suggests that the spleen DC uptake of INPs are independent of CpG concentration under given experimental condition. It should also be noted that for materials in the uptake experiment, INP1q (25% eCpG and 25% Alexa occupancy) were already sufficiently phagocytosed by spleen DCs, while 50% of the U4T portion remained unfunctionalized, which allowed various dosage administrations in a very simple way (i.e., mixing and hybridization of different stoichiometries of ss U4T and ss eCpG). Therefore, these results suggest that systemic in vivo administration of modular INPs may deliver the adjuvant as well as other payloads (e.g., fluorescent probes or other biologically active molecules) to spleen DCs in a general mouse model for immunological study.

2.3.2. Dose-dependent (CpG) immunostimulation

Our observation that systemic administration of INP could induce CpG dose-independent DC uptake prompted us to examine dose-dependent spleen DC activation again in vivo. To determine the dosage effect of CpG in terms of immunostimulation, INPs differed in the amount of hybridized eCpGs were individually injected i.v. to C57BL/6 mice. Twenty four hours after the injection, the spleen DCs of the mice were analyzed. The administration of the INP family (1q, 2q and 4q) led to notable increases in the proportion and number of spleen DCs, which were identified as lineages CD11c+ cells, whereas U4T alone exhibited no effect (Fig. 4a). Moreover, the administration of INP2q and INP4q induced a significantly increase in all the surface levels of CD40, 80, 86, MHC class I, and MHC class II in spleen DCs, while INP1q showed a comparably weak effect for spleen DC activation (Fig. 4b). These data indicate that spleen DC activation is dependent on eCpG coverage on INPs. More specifically, INP2q with merely 50% of U4T hybridized with eCpG already induced full activation of spleen DCs in our immune stimulation experiment. The optimum dosage for in vivo DC activation by INP2q found here was ca. 25 μg of CpG segment (40.8 μM) per injection, which is equal to 1.2 mg/kg.

The CpG-induced immunostimulation via INP delivery was further confirmed by phagocytosis and control experiment. First, to evaluate that INP-phagocytosed DCs were activated, to C57BL/6 mice was injected Alexa-labeled INP2q and analyzed co-stimulatory molecule expression in the Alexa488+ and Alexa488+ spleen CD11c+ DCs after 6 h Alexa488+ CD11c+ spleen DCs expressed significantly higher levels of CD40, 86 and MHC class II compared to Alexa488+ CD11c+ spleen DCs (Fig. S7). Secondly, in eCpG sequence possessing ODN 1826, the immunostimulating sequence (20 nt) were replaced by ODN 2138 (a negative control of ODN 1826). The negative control was then hybridized with U4T at the equimolar concentration of INP2q. As shown in Fig. S8, NP bearing the TLR9 negative sequence (2138-NP) did not induce up-regulation of CD40, 86 and MHC class II in the spleen DCs, whereas INP2q promoted substantial up-regulation of those molecules in the spleen DCs. Therefore, these result suggest that the activated DCs were mediated by the very CpG (ODN 1826) coexisting with the INPs throughout delivery and DC phagocytosis.

2.3.3. Cytokine production

The activation of DCs consequently produces pro-inflammatory cytokine production. To determine whether INP affects the production of pro-inflammatory cytokines, we injected i.v. with INP2q to C57BL/6 mice and analyzed the mRNA levels of pro-inflammatory cytokines in spleen, lung and liver and concentration of the cytokines in the blood serum after 4 and 24 h, respectively. Four hours after INP treatment, we found that the
administration of INP caused a marked increase in mRNA levels of interleukin (IL)-6, IL-12p40, and tumor necrosis factor (TNF)-α in spleen, lung, and liver compared to those of PBS-treated mice (Fig. 5a and Fig. S9). Consistent with increased mRNA levels in the tissue by INPs, serum concentrations of IL-6, IL-12p70, and TNF-α were also significantly increased by the INP injection compared to those treated with PBS (Fig. 5b).

Moreover, 24 h after INP administration, INP treatment led to two, three, and four times increased percentages of TNF-α-, IL-12p70-, and IL-6-producing spleen DCs compared to those treated with PBS, respectively (Fig. 5c and d). From these experiments, we concluded that the systemic administration of INP markedly induced the production of pro-inflammatory cytokines in vivo and spleen DCs were contributed the production of those cytokines in response to INPs.

In order to elucidate the effect of confining the CpG motif within the nanoparticle system for DC activation, we performed a set of in vivo experiments, including the injection of INP2q and the same amount of pristine eCpG and CpG. The C57BL/6 mice were treated i.v. with eCpG, CpG or INP at the same concentration of CpG (40.8 μM), and the outcome was analyzed after 24 h. While eCpG and CpG induced moderate up-regulation of CD80, CD86, and MHC class II as the immunostimulatory ligand compared to PBS, INP2q induced much higher expression levels of the co-stimulatory molecules compared to two oligonucleotides alone controls (Fig. S10a and b). Furthermore, the serum concentrations of pro-inflammatory cytokines in the INP-treated mice were significantly higher than those treated with pristine eCpG and CpG (Fig. S10c). This difference of spleen DC activation might be caused by confined size of INP during DC uptake [33] since an INP presents multiple CpG segments on its surface [24]. In previous investigations, it was found that up to four CpGs exposed on a DNA
nanostructure led to pronounced DC activation compared to pristine CpG [24]. Therefore, our data suggest that incorporation of the CpG motif into a DNA nanoparticle and a high surface density of the immune stimulatory sequence induce a strong DC activation \textit{in vivo}.

2.3.4. INP versus tetrahedron bearing CpG

The increased cytokine production followed by effective DC activation, as shown above, motivated us to examine the influence of the nature of the carrier for the delivery of immunostimulatory CpG. We chose a DNA tetrahedron (Td) nanoparticle since the size of Td (6 nm) is close to our INP, and CpG-mediated DC activation \textit{in vitro} was reported before by Li et al. [24]. First, we prepared a DNA tetrahedron, which contains four CpG sequences protruding from each vertex named Td-CpG. This nanoobject was constructed

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Fig. 3. Spleen DCs take up INP \textit{in vivo}. C57BL/6 mice were injected i.v. with Alexa488-labeled micelles (INP-Alexa) for 2 h. (a) Uptake of INP-Alexa by splenocytes was analyzed using flow cytometry (left panel). Phagocytosis of INP by spleen DCs was measured. Spleen DCs were defined as lineage (CD3, Thy1.1, B220, Gr1, CD49b, TER-119) and CD11c+ cells (right panel). Numbers in the plots show the percentage of the cells in the respective quadrant among the total cells shown in the plots. (b) Mean of Alexa488 positive cell percentages in spleen DCs. (c) Composite confocal representative images of spleen sections 2 h after INP-Alexa administration showing fluorescence signals of Alexa488 (green), CD11c (red), TLR9 (blue) and nuclear stain with DAPI (black and white), respectively. Low magnification (scale bar, 200 μm) and higher magnification of the boxed area are presented (right panel). (d) C57BL/6 mice were injected i.v. with or without ODN 2088. One hour after ODN 2088 injection, mice received with INP-Alexa for 2 h. Uptake of INP-Alexa by spleen DCs was analyzed using flow cytometry (left panel). (e) Mean of Alexa488 positive cell percentages in spleen DCs (right panel). Mean ± SEM (n = 6 [b and e]). **, p < 0.01 by Student’s t-test. Data are representative of 6 independent samples (n = 6 mice [a, c and d], total 2 independent experiments). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
from four 80 nt long oligonucleotides (see Table S2 for the sequences composition). We performed identical DC activation and pro-inflammatory cytokine production in serum for mice individually treated with Td-CpG, INP1q, and INP2q. Here it should be noted that Td-CpG was administered at 5 μM after the dilution of stock solution (10 μM), which is the experimental upper limit of the
In stark contrast, levels of pro-inflammatory cytokines produced by the same amount of CpGs present on the nanoparticle surface (Fig. 6). When administering Td-CpG and INP1q, we observed a significant effect of CpG quantity both on DC activation and cytokine production. Both Td-CpG and INP1q showed moderate DC activation and cytokine production with the same amount of CpGs present on the nanoparticle surface (Fig. 6). In stark contrast, INP2q yielded a much higher up-regulation of the co-stimulatory molecule in spleen DCs and significantly elevated levels of pro-inflammatory cytokine in the serum of INP2q-treated mice compared to those treated with Td-CpG (Fig. 6). The finding that doubling the density of CpGs on the surface of the nanoparticles (Td-CpG and INP1q vs. INP2q) resulted in a more than twofold increase of DC activation and cytokine levels suggests that there is a threshold density of CpG on the surface of such nanoparticles. Based on our aggregation number (Z) calculation of analogous DNA nanoparticles (Z = 25 ± 2), it turned out that the threshold density of CpG per particle required for maximum immunostimulation is above 12 CpG motifs.

Moreover, these experiments demonstrate the power of DNA-lipid-based nanoparticle systems for immunostimulation. While wireframe nanoobjects constructed via Watson-Crick base pairing, such as DNA tetrahedron, hold a few ligands on the surface, compact DNA nanoparticles composed of ss DNA amphiphiles, such as lipid-DNA, easily allow the decoration of multiple CpG ligands within a confined small volume. Another striking feature of DNA amphiphile-based carriers for immunostimulation is that they are composed of a small number of nucleotides compared to other DNA nanoobjects, such as DNA tetrahedron. This allows high yield synthesis by solid phase methods and facile upscaling. Considering the exponential decrease of yield when increasing the lengths of oligonucleotides during solid phase synthesis, these features are very relevant in case of preclinical testing when larger quantities of nucleic acid materials are needed. Although there are studies demonstrating that CpG can induce spleen cDC activation [34,35], the effect of CpG-conjugated DNA nanoobjects into spleen DC in vivo has not well been characterized. Uno et al., has examined the immune stimulatory effect of CpG DNA-nanoassemblies, however, most experiment has been performed in vitro using murine macrophage cell line RAW264.7 cells, mouse bone marrow-derived DCs (BMDCs) and isolated spleen macrophages [23,36]. Although the studies showed that injection of CpG DNA nanoassemblies promotes IL-12p40 expression in serum, successful delivery of the CpG-nanoassemblies to the immune cells in vivo has not been well addressed. Moreover, activation of spleen DCs or macrophage by CpG-nanoassemblies in vivo has not been studied [23,36]. Therefore, the studies need to be systematically examined in vivo. In line with the studies, INPs were also successfully delivered to cytoplasmic TLR9 in spleen DC and induced activation of spleen DC. Furthermore, INPs not only deliver adjuvants to the spleen DCs, but also can also load AGs. This is very important because particular DCs, that simultaneously obtained both adjuvant and AG, will induce subsequent AG-specific immune response to fight against the corresponding pathogen with minimized side effect which might be caused by non-specific immunostimulation.

3. Conclusion

In this study, we demonstrated that lipid-DNA-based nanoparticles are a biocompatible, simple and scalable delivery platform for immunostimulation, which effectively promote spleen DC activation. Furthermore, our extended in vivo immunology results support the notion that the systemic administration of INP efficiently promotes the up-regulation of co-stimulatory molecules and the production of pro-inflammatory cytokines. More importantly, our lipid-DNA nanoparticle half-filled with the CpG segment (INP2q) has already fully induced the activation of spleen DCs, while the similarly sized DNA tetrahedron can only be loaded with fewer CpG strands with limited immunostimulation. Our findings

Fig. 6. Comparison of spleen DC activation after injection of CpG functionalized DNA tetrahedron (Td-CpG) and INPs. C57BL/6 mice were injected i.v. Td-CpG (12.5 μg), INP1q (12.5 μg) or INP2q (25 μg) and waited for 24 h. Numbers in μg denote the quantity of CpG segment per injection. (a) Expression levels of CD40, CD80, CD86 in spleen DCs were measured by flow cytometry and MFI of these molecules is shown. (b) IL-6, IL-12p70 and TNF-α concentrations in sera are shown. Mean ± SEM (n = 6 [a and b]). *p < 0.05; **p < 0.01; ***p < 0.001 by Student’s t-test.
also indicate that the INPs still have free hybridization sites available to be administered with other molecules, such as cancerous AGs or imaging reagents like fluorescently labeled DNAs, as demonstrated herein. Since effective immunotherapy requires AG-specific immune activation, INP-AG formation via straightforward DNA duplex formation might be a suitable functionalization strategy and a potential platform for cancer or infectious disease immunotherapy. In the future, based on our in vivo INP results herein, we will further investigate INP-AG systems to promote AG-specific immune responses in the realm of cancer vaccination.

4. Experimental section

4.1. INP preparation and injection

From a stock solution of FPLC-purified U4T by anion-exchange (Hi-Trap Q HP, GE Healthcare), INP solutions were prepared to adjust the final concentration of U4T to 80 μM U4T was hybridized with the desired amount of either eCpG (Bioneer Co., Korea) or fluorescently labeled cU4T (Bioneer Co., Korea) by thermal annealing (80–20 °C for 60 min) in the presence of PBS (5 mM). For an i.v. injection, 100 μL of each prepared INP solution was used per mouse.

4.2. Mice

C57BL/6 mice (6 weeks old) were obtained from the Shanghai Public Health Clinical Center and kept under pathogen-free conditions. The mice were maintained in a room with controlled temperature (20–22 °C), humidity (50%−60%), and light (12 h: 12 h), with free access to standard rodent chow and water. All experiments (Table S1) were carried out under the guidelines of the Institutional Animal Care and Use committee at the Shanghai Public Health Clinical Center. The protocol was approved by the committee on the Ethics of Animal Experiments of the Shanghai Public Health Clinical Center and kept under pathogen-free conditions. The mice were sacrificed by CO2 inhalation euthanasia, and all efforts were made to minimize suffering.

4.3. Antibodies

Isotype control antibodies (ABs) (IgG1, IgG2a, or IgG2b), CD11c (N418), CD40 (3/23), CD80 (16–10A1), CD86 (GL-1), anti-IL-6 (MP5-XT22) were purchased from eBioScience Inc.) for 30 min. Control staining with isotype control isotype-matched irrelevant mABs were incubated with anti-cytokine antibodies in Perm/Wash buffer and then labeled with B220 (RA3-6B2), anti-Gr-1 (RB68C5), anti-CD49b (DX5), and anti-TER-119 (TER-119). The lineage-CD11c + cells were defined as spleen DCs. Analysis was carried out on a FACS Aria II (Becton Dickinson Biosciences).

4.4. Flow cytometry analysis

Cells were washed with PBS containing 0.5% bovine serum albumin, preincubated for 15 min with unlabeled isotype control ABs, and then labeled with fluorescently labeled ABs by incubation on ice for 30 min followed by washing with PBS. Cells were analyzed on a FACS Aria II (Becton Dickinson Biosciences) and with FlowJo 8.6 software (FlowJo LLC). Cellular debris was excluded from the analysis by forward- and side-scatter gating. Dead cells were further excluded by 7 aminoactinomycin D (7AAD) (BioLegend Inc.) staining and gating on the 7AAD-negative population. As a control for nonspecific staining, isotype-matched irrelevant mABs were used.

4.5. BMDC differentiation

The initial cultures were prepared as described previously[37,38]. Bone marrow nucleated cells (1 × 10^6 cells/mL) were cultured in 5 mL modified RPMI 1640 medium containing 10% FBS in 6 well plates: 50 ng/mL rmGM-CSF plus 50 ng/mL rmIL-4 were added to the medium to support the generation of BMDCs. Unless otherwise stated, the cells were cultured for 6 days at 37 °C under 10% CO2. The cultured cells were washed twice in fresh medium before the additional experiments were conducted. Differentiation of BMDCs were defined by CD11c expression on a flow cytometry.

4.6. Spleen DC analysis

Spleen DCs were analyzed as described elsewhere[39–42]. Briefly, the tissues were cut into small fragments and digested, with 2% FBS containing collagenase for 20 min at room temperature. Cells from the digest were centrifuged to a pellet, and the pellet was resuspended in 5 mL of a 1.077 histopaque (Sigma-Aldrich Co. LLC.). An additional 5 mL of histopaque was layered below, and culture medium was layered above the cell suspension, which was then centrifuged at 1700 g for 10 min. The light density fraction (<1.077 g/cm3) was collected and incubated for 20 min with the following FITC or PE-conjugated monoclonal antibodies (mABs): anti-CD3 (17A2), anti-Thy1.1 (OX-7), anti-B220 (RA3-6B2), anti-Gr-1 (RB68C5), anti-CD49b (DX5), and anti-TER-119 (TER-119). The lineage-CD11c + cells were defined as spleen DCs. Analysis was carried out on a FACS Aria II (Becton Dickinson Biosciences).

4.7. ELISA

IL-6, IL-12p70, and TNF-α concentrations in the sera were measured in triplicate using standard enzyme-linked immunosorbent assay (ELISA) kits (BioLegend Inc.).

4.8. Real-time PCR

Total RNA was reverse-transcribed into cDNA using oligo (dT) primer and M-MLV reverse transcriptase (Promega Co.). The cDNA was subjected to real-time PCR amplification (Qiagen AG) for 40 cycles with annealing and extension temperature at 60 °C, on a LightCycler 480 Real-Time PCR System (Roche AG). See Table S2 for the primer sequences.

4.9. Intracellular cytokine staining

As previously described in detail[37], single cells prepared from spleen were stimulated in vitro for 4 h with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Calbiochem) and ionomycin (1 μM; Calbiochem), with the addition of monensin solution (Biolegend) during the final 2 h. For intracellular cytokine staining, cells were stained for surface molecules first, then fixed and permeabilized with Cytofix/Cytoperm buffer (eBioscience Inc.) and subsequently incubated with anti-cytokine antibodies in Perm/Wash buffer (eBioscience Inc.) for 30 min. Control staining with isotype control IgGs was performed in all experiments.

4.10. Statistical analysis

All results with statistical analysis are expressed as the mean ± standard error of the mean (SEM). The statistical significance of differences between experimental groups was calculated using analysis of variance with a Bonferonni post-test or an unpaired Student’s t-test. All p-values < 0.05 were considered significant. All the data here are the mean ± SEM. *p < 0.05, ***p < 0.001 versus PBS group.
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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.11.020.

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