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Intertwining DNA-RNA nanocapsules loaded with tumor neoantigens as synergistic nanovaccines for cancer immunotherapy

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Nanomedicines that co-deliver DNA, RNA, and peptide therapeutics are highly desirable yet remain underdeveloped for cancer theranostics. Herein, we report self-assembled intertwining DNA-RNA nanocapsules (iDR-NCs) that efficiently delivered synergistic DNA CpG and short hairpin RNA (shRNA) adjuvants, as well as tumor-specific peptide neoantigens into antigen presenting cells (APCs) in lymph nodes for cancer immunotherapy. These nanovaccines were prepared by (1) producing tandem CpG and shRNA via concurrent rolling circle replication and rolling circle transcription, (2) self-assembling CpG and shRNA into DNA-RNA microflowers, (3) shrinking microflowers into iDR-NCs using PEG-grafted cationic polypeptides, and (4) physically loading neoantigen into iDR-NCs. CpG and shRNA in iDR-NCs synergistically activate APCs for sustained antigen presentation. Remarkably, iDR-NC/neoantigen nanovaccines elicit 8-fold more frequent neoantigen-specific peripheral CD8+ T cells than CpG, induce T cell memory, and significantly inhibit the progression of neoantigen-specific colorectal tumors. Collectively, iDR-NCs represent potential DNA/RNA/peptide triple-co-delivery nanocarriers and synergistic tumor immunotherapeutic nanovaccines.
Natural nucleic acids (e.g., DNA, RNA) have inspired the development of nucleic acid nanotechnology and synthetic functional nucleic acids. Specifically, inspired by the abilities of natural nucleic acids to structurally code and store genetic information, nucleic acid nanotechnology utilizes synthetic nucleic acids to precisely engineer nanostructures.

Nucleic acid nanotechnology has enabled the development of various nucleic acid nanomedicines for biomedical applications, including drug delivery. Furthermore, inspired by the biological functions of natural nucleic acids, synthetic functional nucleic acids have been developed as therapeutics and targeting ligands. For instance, unmethylated cytosine-guanine oligodeoxynucleotides (CpG) have been extensively investigated as immunomodulatory cancer therapeutics. CpG triggers the Toll-like receptor 9 (TLR9) pathway to activate APCs, such as dendritic cells (DCs) and macrophages. In addition, RNA therapeutics, such as shRNA, are another class of nucleic acid therapeutics that precisely regulate gene expression via RNA interference.

Empowered by the inherent structure programmability and versatile functionalities of nucleic acids, nucleic acid nanotechnology enables engineering of multiple functional nucleic acids into a single nanomedicine, which is especially of interest in co-delivering multiple synergistic nucleic acid therapeutics for combination cancer therapy. Towards this end, a simple technology to construct nucleic acid nanostructures that incorporate both DNA and RNA therapeutics is highly desirable, yet remains an underdeveloped field of study. Previously, nucleic acid nanostructures have been constructed via nucleic acid hybridization, and recently via co-crystallization of nucleic acids with inorganic supplements during rolling circle replication (RCR) and rolling circle transcription (RCT) to respectively construct DNA or RNA nanostructures. However, these technologies are either limited by complicated design, limited biostability, or most importantly, the lack of ability to simply incorporate both DNA and RNA therapeutics into the same nanomedicine for co-delivery.

Cancer immunotherapy has made encouraging breakthroughs in the past decades, which modulates the immune system to treat cancer. Because the immune system in cancer patients is compromised by multi-tier immunosuppressive signaling pathways, combination therapy is especially significant for cancer immunotherapy by combining synergistic therapeutics to modulate multiple pathways, for example, activating anticancer immunostimulatory pathways and deactivating...
immunosuppressive pathways. As mentioned, CpG has shown encouraging clinical efficacy in cancer treatment by activating the immunostimulatory TLR9 pathway\(^{20}\). Moreover, Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathways have been shown as promising targets for cancer immunotherapy\(^{21}\). STAT3, a member of the STAT family, is immunosuppressive in APCs by multiple mechanisms such as inducing antigen-specific T cell tolerance\(^{22-24}\). Even worse, STAT3 suppresses CpG-activated immunostimulation\(^{25}\), suggesting the clinical demand to synergistically activate TLR9 pathway and disarm STAT3 pathway in APCs for combination cancer immunotherapy\(^{26-28}\). Towards this end, therapeutic CpG and Stat3 shRNA are promising, yet the clinical applications of these therapeutics have thus far been hindered by: (1) limited in vivo nucleic acid biostability (e.g., nuclease susceptibility) or otherwise costly chemical modification to improve biostability, (2) suboptimal pharmacodynamics and pharmacokinetics that result in rapid clearance and inefficient delivery in vivo, (3) inefficient intracellular nucleic acid delivery due to negative charges, (4) limited therapeutic efficacy of monotherapy, and (5) toxicity of many current nucleic acid delivery vehicles. Nanovaccines, owing to typically efficient delivery into secondary lymphoid organs such as lymph nodes (LNs), have been enthusiastically explored for delivering immunomodulatory adjuvants and antigens for cancer immunotherapy\(^{29-34}\), which together can elicit potent and durable adaptive immunity. While previous investigation of tumor-associated antigens has yielded moderate therapeutic efficacy with risks of side effects associated with autoimmune responses, tumor-specific neoantigens have emerged as a promising alternative for potent and safe immunotherapy. Preclinical and clinical evidences have supported the key role of neoantigens in tumor immunotherapy\(^{35-37}\). Unlike
tumor-associated antigens that are expressed in both tumors and healthy tissues, neoantigens, which are derived from somatic mutations in tumors, are expressed exclusively in tumor cells but not in healthy cells. Therefore, the use of neoantigens as vaccine components would potentially avoid autoimmunity against healthy tissues, and nanovaccines that co-deliver adjuvants and neoantigens are of tremendous interest for tumor immunotherapy.\(^1\)\(^2\)\(^3\)

Herein, we developed biostable iDR-NC/neoantigen complexes as nanovaccines that incorporated CpG and Stats3 shRNA, as well as tumor-specific neoantigens for efficient co-delivery and immunomodulation in cancer immunotherapy. Unlike DNA and RNA nanostructures that were previously constructed via separate RCR or RCT\(^5\),\(^6\), iDR-NCs are hybrid DNA-RNA nanostructures that are generated via combined RCR and RCT in the same reaction system, which essentially incorporates both DNA and RNA therapeutics into the single nanostructures. Moreover, given the large sizes of microstructures constructed from RCT or RCR (\(d \approx 0.5 - 5 \mu m\)), it is essential to shrink them for efficient drug delivery. Although polyethyleneimine (PEI) can meet this end, the cytotoxicity of highly cationic PEI raises serious safety concern.\(^16\) In this study, we synthesized biocompatible PEG-grafted polypeptide (PPT-g-PEG) copolymers to shrink the microflowers (MFs) constructed from combined RCR/RCT. Specifically, PPT-g-PEG imparts multiple functions to precisely engineer nanovaccine delivery: (1) the cationic PPTs transform MFs into NCs to enhance delivery efficiency to LNls at the tissue level and to APs at the cellular level, (2) acid-labile PEG not only ensures high solubility of copolymer and thus effective MF shrinkage, but also enhances the proton sponge effect to promote intracellular delivery after PEG shedding and hence exposure of cationic PPTs in acidic endolysosomes, (3) the hydrophobic PPTs allows loading of tumor-specific neoantigens into iDR-NCs via hydrophobic interactions between peptide neoantigens and PPTs for co-delivery of adjuvants and antigens, and (4) biocompatible and biodegradable PPT-g-PEG is expected to have good safety profile. The resulting iDR-NCs synergistically activated APCs, and iDR-NC/neoantigen complexes elicited potent and durable tumor-specific antitumor immunity for tumor immunotherapy.

**Results**

**Self-assembly of iDR-NCs as nanoadjuvants.** iDR-NCs were constructed in four steps: (1) Two DNA templates were designed to encode CpG 1826 (sequence: TCCATGACGTTCCT-\(\cdots\)GAGCTT-GACGGT and Stats3 shRNA, respectively; (2) The reaction condition was optimized to allow concurrent RCR and RCT in the same solution; (3) Combined RCR and RCT produced tandem CpG and shRNA, which were subsequently self-assembled into hybrid CpG-shRNA MFs; and (4) MFs were shrunk by positively-charged PPT-g-PEG copolymers to form iDR-NCs (Fig. 1). Specifically, we designed a linear phosphorylated DNA template and a DNA primer for RCR to generate tandem CpG, and a linear phosphorylated DNA template for RCT to produce tandem shRNA (Supplementary Table 1). CpG template was circularized using the DNA primer and T4 DNA ligase, and the shRNA template was circularized by CircLigase (Supplementary Fig. 1). To allow RCT and RCR in the same reaction, we optimized the reaction buffer and temperature which permitted efficient RCR (typically in RCR buffer at 30 °C) and RCT (typically in RCT buffer at 37 °C) (Supplementary Table 2). It was found that RCT, but not RCR, was susceptible to change in reaction temperature and buffer. In RCT buffer at 37 °C, RCR was still highly efficient, so this condition was chosen for combined RCR/RCT to efficiently produce nucleic acids (Fig. 2a). Altogether with magnesium pyrophosphate (MgPPi), the generated CpG and shRNA were self-assembled into CpG-shRNA MFs after 24 h reaction, as shown by SEM (Fig. 2b) and XRD analysis (Supplementary Fig. 2).

To shrink the above MFs to afford nanovaccines, PEGylated cationic polypeptide PPT-g-PEG was designed and synthesized, with the expectation of less cytotoxicity than conventional nucleic acid delivery polymers such as PEI. PPT-g-PEG was synthesized with PPTs of 45 repeating units of modified L-glutamic acids that contained 90 units of primary amines, and a series of acid-responsive-cleavable PEG grafts. The PEG was grafted onto PPTs via an acid-labile Schiff base linker. PEGylation was expected to enhance the solubility, biocompatibility, and antifouling efficacy of PPT-g-PEG. Upon PEG cleavage in acidic endolysosome, PEG shedding would expose cationic PPTs to further enhance endosome escape.\(^38\) To synthesize PPT-g-PEG (Supplementary Fig. 3), L-glutamic acid was first modified to introduce an alkyne group using propargyl alcohol, and then triphosgene was used to transform it into N-carboxyanhydride (NCA) monomers.\(^39\)\(^40\) PPT was synthesized by controlled ring-opening polymerization of NCA monomer via an accelerated nitrosonium flow method.\(^39\) After complete consumption of the monomer as determined by \(^1\)H NMR, the polymer was purified by three times of precipitation into diethyl ether. Gel permeation chromatography showed monomodal molecular weight distribution with a low polydispersity index of 1.23 (Supplementary Fig. 4). Next, the pedant alkyne group was transformed into positively-charged amine via an efficient thiol-yne reaction with 2,2-dimethoxy-2-phenylethanolironen as a radical initiator under UV light. Subsequently, PEGylation was achieved by forming a Schiff base between the primary amine of PPTs and aldehyde from PEG-CHO. All structures were confirmed by \(^1\)H NMR (Supplementary Figs. 5–10).

The next step to construct iDR-NCs was the shrinkage of CpG-shRNA MFs using the above PPT-g-PEG (Fig. 1). To screen PPT-g-PEG for optimal shrinkage, CpG-shRNA MFs were respectively incubated with PPT-g-(PEG)\(_6\), PPT-g-(PEG)\(_{12}\), PPT-g-(PEG)\(_{18}\), and PPT-g-(PEG)\(_{24}\) that all had PPT backbones of 45 repeating units of modified L-glutamic acids with 6, 12, 18 and 24 copies of acid-labile PEG grafts, respectively. Note that adding one copy of PEG would reduce one positive charge in the copolymer. PPT-g-(PEG)\(_6\) with non-cleavable PEG served as a control. 48 h after incubating MFs with 2.5 mg/mL polymers in PBS, iDR-NCs were purified by centrifugation to remove free polymers. SEM revealed that PPT-g-(PEG)\(_6\) most effectively shrunk the MFs to iDR-NCs, whereas PPT-g-PEG with 12, 18 and 24 PEG copies induced only partial shrinkage at best (Fig. 2c, Supplementary Fig. 11) likely because of insufficient positive charges and because crowded PEG grafts hindered the interaction between PPTs and MFs. Using iDR-NCs labeled with Alexa 488 via the DNA primer (Supplementary Fig. 12), flow cytometry suggested that PPT-g-(PEG)\(_6\) yielded the most efficient uptake of polymer-incubated MFs by RAW264.7 macrophages and DC2.4 cells (Supplementary Fig. 13). PPT-g-(PEG)\(_6\) was then selected for the following studies. Neither PPT-g-(PEG)\(_6\) nor PPT controls displayed NC-like structures, and PPTs without PEG failed to shrink MFs likely due to limited solubility (Supplementary Fig. 14). In a polymer concentration-dependent manner, MF shrinkage was complete with at least 2.5 mg/mL PPT-g-(PEG)\(_6\) (Supplementary Fig. 15), which was therefore used in the following studies. The process of NC formation was monitored by SEM at a series of time points after incubating MFs with PPT-g-(PEG)\(_6\). Multiple iDR-NCs gradually “bubbled” out from the outer layers of each MF at early stage, and one MF were thus shrunk to multiple iDR-NCs within a span of up to 2 days (Fig. 2d). The diameters were reduced from 1272 ± 159 nm for MFs to 252 ± 23 nm for iDR-NCs (Fig. 2e), and the zeta potential was increased from −39 mV for MFs to
1.5 mV for iDR-NCs due to the positive charge of PPT-g-(PEG)$_6$ (Supplementary Fig. 16). iDR-NCs were further characterized by atomic force microscopy (AFM), transmission electron microscopy (TEM), and high-resolution TEM (HR-TEM) (Supplementary Fig. 17). The interior structure of iDR-NCs was studied using focused ionization beam (FIB) that cut iDR-NCs to expose the hollow interior structures of iDR-NCs, as revealed by SEM (Fig. 2f). To estimate the yield of CpG and shRNA in iDR-NCs, CpG MFs, and shRNA MFs were generated in separate RCR and RCT under the same conditions as mixed RCR/RCT, and the resulting MFs were washed to remove residual substrates and then EDTA-treated to dissolve Mg$_2$PPI (Supplementary Fig. 18). By measuring absorbance at 260 nm, CpG template was estimated to be replicated for ca. 12 times and shRNA for ca. 4 times in iDR-NCs, under the assumption that all nucleic acids in MFs were shrunk into iDR-NCs. Importantly, PPT-g-PEG and iDR-NCs were significantly less cytotoxic than PEI in RAW264.7 macrophages, demonstrating the biocompatibility of PPT-g-(PEG)$_6$ (Fig. 2g). Moreover, iDR-NCs showed high biostability even when incubated in 10% serum for 5 h (Supplementary Fig. 19).

**Efficient intracellular delivery of iDR-NC nanoadjuvants.** As TLR9 (receptor for CpG) resides in the endolysosomes of APCs and Stat3 mRNA is in the cytosol, efficient intracellular delivery of iDR-NCs is essential for potent immunomodulation. After Alexa 488-labeled iDR-NCs were incubated with RAW264.7 macrophages and DC2.4 cells for 3 h, efficient intracellular delivery of iDR-NCs was observed by confocal microscopy (Fig. 3a, b) and flow cytometry (Fig. 3c). Specifically, confocal microscopy revealed massive colocalization of internalized iDR-NCs with the endolysosome stained by LysoTracker Red, and owing to shrinkage, the delivery efficiency of iDR-NCs dramatically outperformed the parent MFs (Fig. 3a, b). Confocal microscopy with 3D deconvolution clearly revealed iDR-NCs in the endolysosome of DC2.4 cells, which would allow binding of CpG with TLR9 for immunostimulation (Fig. 3d; Supplementary Fig. 20; Supplementary Movie 1). As shRNA and CpG were co-incorporated into iDR-NCs, these results suggest efficient intracellular delivery of both CpG and shRNA. We hypothesize that iDR-NCs would dissociate in the acidic endolysosome to liberate CpG, and the acid-labile linker in PPT-g-(PEG)$_6$ would break in acidic endolysosome so that the exposed cationic PPTs in iDR-NCs would facilitate the endosomal escape of shRNA via proton sponge effect. Overall, these results provide the basis for intracellular immunomodulation by iDR-NC nanovaccines.

**iDR-NC/antigen co-delivered adjuvants and antigens to APCs.** Co-delivery of adjuvants and antigens into APCs is key for optimal antigen presentation. To study this, we used Alexa 555-labeled iDR-NCs and model antigen CSIINFEKL, an epitope of chicken ovalbumin (OVA) with a cysteine appended on the N-terminal. To monitor the behaviors by fluorescence, CSIINFEKL was modified with FITC on lysine that was reported not to affect its binding with major histocompatibility complex I (MHC I)$.^41$ CSIINFEKL was loaded into iDR-NCs through physical complex driven by hydrophobic interactions between CSIINFEKL and PTTs. Specifically, iDR-NCs (from 200 µL RCT/RCT products) were co-incubated with CSIINFEKL$_{FITC}$ (100 µM) at room temperature for 5 h, followed by washing with PBS and centrifugation for three times to remove free antigen. As determined by the FITC fluorescence of unloaded antigens, the amount of CSIINFEKL loaded into the resulting iDR-NC/CSIINFEKL$_{FITC}$ complexes (SEM images in Supplementary Fig. 17).
Intracellular co-delivery in DCs was then investigated by super resolution fluorescence imaging using a home-built instant structured illumination microscope (instant SIM) that enables the high-speed acquisition of super resolution images with relatively little photobleaching (which was critical in imaging the easily photobleached FITC)42. Specifically, DC2.4 cells were incubated with iDR-NC-Alexa 555/CSIINFEKL complexes for 6 h, then washed before super resolution imaging. Instant SIM revealed a high degree of colocalization of iDR-NCs with CSIINFEKL in DCs (Fig. 4a), suggesting that iDR-NCs is co-delivered with antigen and providing the basis for efficient antigen presentation. To examine antigen presentation, bone marrow derived dendritic cells (BMDCs) were incubated with iDR-NCs/CSIINFEKL complexes for a total of 6, 24, and 48 h before confocal microscopy observation. For 24-h and 48-h points, cells were washed at 14 h after adding iDR-NCs/CSIINFEKL, followed by further incubation to investigate the sustainability of antigen presentation. Compared to free CpG control, iDR-NCs not only enhanced the uptake of CSIINFEKL but also resulted in sustained presence of CSIINFEKL on BMDC surfaces, likely resulting from sustained antigen presentation of CSIINFEKL (Fig. 4b). Note that, at early stage, both free CSIINFEKL and CSIINFEKL loaded in iDR-NCs were efficiently internalized into BMDCs and presented on BMDC surfaces, likely due to efficient uptake of particulate CSIINFEKL (formed by hydrophobic interactions) as well as strong epitope binding of CSIINFEKL with MHC I on BMDC surfaces.

Synergistic in vitro immunoactivation by CpG/shRNA iDR-NCs. We studied Stat3 silencing in DC2.4 cells that were treated with iDR-NCs for 48 h. Real-time quantitative RT-PCR of mRNA from the treated cells demonstrated the specific silencing of Stat3, compared with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 5a). Treated DCs were further analyzed for STAT3 phosphorylation at tyrosine 705 (Y705), a prerequisite of STAT3 activation via dimerization, translocation into nucleus, DNA binding, and terminal differentiation and growth arrest in monocytes in response to cytokine stimulation. Specifically, the as-treated DC2.4 cells were permeabilized and intracellularly stained using an antibody against phosphorylated STAT3 (p-STAT3). Lipofectamine-transfected Stat3 siRNA and Stat3 inhibitor, Stattic, served as controls. Flow cytometry suggested that iDR-NCs downregulated p-STAT3 (Fig. 5b), at higher efficiency than siRNA, likely due to efficient delivery and bio-compatibility of iDR-NCs relative to Lipofectamine.
immunostimulation (Fig. 5c). Consistently, ELISA showed that, compared with CpG or CpG MFs, treatment with iDR-NCs significantly enhanced the secretion of TNFα, IL-6, and IL-12p40 than CpG or CpG MFs, after 24 h. Stat3 siRNA alone was used as a control. (ns: non-significant; *p < 0.05; **p < 0.01; n = 3; one-way ANOVA with Bonferroni post-hoc test). Data represented as mean ± s.e.m.

Delivery of iDR-NCs to LNs and LN APCs in mice. We then studied the in vivo delivery and immunostimulation of iDR-NCs in immunocompetent C57BL/6 mice. Locally administered nanovaccines exploit the physiological characteristics of lymphatic drainage to transport nanovaccines via lymphatics to LNs, where various lymphocytes reside and immune responses are orchestrated. The sizes of iDR-NCs fell within the nanoparticle size range that allow minimal systemic dissemination and efficient lymphatic drainage for LN accumulation31, which motivated us to study LN delivery of these nanoadjuvants. To this end, iDR-NCs were prepared using NOTA-modified PPT-g-(PEG)₆ and then radiolabeled with ^⁶⁴Cu through NOTA chelator. ^⁶⁴Cu-labeled iDR-NCs were injected subcutaneously (s.c.) at the tail base or ipsilateral to tumor (i.l.t.), respectively, to C57BL/6 mice bearing MC38 syngeneic colon adenocarcinoma tumor (Fig. 6a). I.l.t. injection features proximity with tumor-derived antigens in its tumor-draining LNs (TDLNs). At 1 day post injection, draining inguinal (IN) LNs and axillary (AX) LNs were resected to quantify the radioactivity by γ counting, as well as positron emission tomography (PET). S.c. injection delivered a total of 3-fold more iDR-NCs than i.l.t. injection into IN and AX LNs (Fig. 6b, c). Specifically, the percent injected dose (%ID) of s.c.-injected iDR-NCs was 0.13% in IN LNs and 0.11% in AX LNs. In contrast, only 0.046% of i.l.t.-injected iDR-NCs were delivered to AX TDLNs and a total of <0.02% of iDR-NCs were in IN and AX non-TDLNs (Fig. 6c). Despite the potential of TDLNs to harbor tumor antigens, we chose s.c. injection because of its relatively efficient LN delivery and that exogenous MC38-specific neoantigen was used together with iDR-NCs for antigen-specific immunomodulation in this study.

As mentioned, potent antigen presentation on APC surfaces and effective immunomodulation demand co-delivery of adjuvants and antigens into APCs. We thus studied co-delivery of Alexa 555-labeled iDR-NC/CSIINFEK(FITC)L complexes after s.c. injection at the tail base of C57BL/6 mice. At 1 day post injection, draining IN LNs were resected for flow cytometric analysis of Alexa 555 and FITC signals in B220⁺ B cells, F4/80⁺ macrophages, and CD11c⁺ DCs, which are major populations of APCs that can present antigens to naïve T cells. 18.2% DCs and 25.4% macrophages were Alexa 555⁺FITC⁺, demonstrating efficient co-delivery of iDR-NCs with peptide antigen to LN APCs in vivo (Fig. 6d). In comparison, Alexa 555⁺FITC⁺ B cells were <1%. The efficient in vivo uptake of NCs and antigens was attributed to shrink sizes of iDR-NC/CSIINFEK(FITC)L complexes. Further, at 1 day after iDR-NC immunization, CD80 expression was elevated in LN DCs and macrophages, indicating in vivo APC activation (Fig. 6e). The efficient co-delivery and immunostimulation of iDR-NCs in vivo are expected to elicit potent T cell responses.

![Flow cytometry results showing that p-STAT3 expression was downregulated in DC2.4 cells by siRNA iDR-NCs compared with CpG MFs](image1)

![Real-time quantitative RT-PCR results showing Stat3 silencing in DC2.4 cells treated with iDR-NCs](image2)
iDR-NC/neoantigen potentiated T cell responses with memory. T cell responses against neoantigen-presenting tumor cells play a central role in cancer immunotherapy. To study iDR-NC/neoantigen for neoantigen-specific cancer immunotherapy, we attempted to analyze T cell responses after immunizing C57BL/6 mice with iDR-NC/neoantigen complexes. Neoantigens were generated from somatic tumor mutation and were identified by exome sequencing and mass spectrometry. We used neonantigen Adpgk generated from mutation (ASMTNRELM → ASMTNEMLM) in MC38 tumor27. Again, iDR-NC/neoantigen complexes (SEM images in Supplementary Fig. 21b) were prepared by co-incubating iDR-NCs with hydrophobic Adpgk to allow physical complexation of Adpgk with the PPT moieties via hydrophobic interactions. By quantifying the unloaded Adpgk using UV absorbance (280 nm), the amount of Adpgk loaded into iDR-NCs was 2.7 molar equivalents of CpG (CpG: Adpgk = 1:2.7). Somatic C57BL/6 mice were immunized with CpG/shRNA<i>Stat3</i> iDR-NC/Adpgk complexes (2 nmol CpG equivalents, 17 μg Adpgk) on day 0 for priming and day 14 for boosting. On day 21, peripheral blood was collected, red blood cells lysed, and CD8<sup>+</sup> T cells in peripheral blood mononuclear cells (PBMCs) were stained using a PE-conjugated H-2Db-ASMTNMELM dextramer. This dextramer is a molecular complex that mimics MHC I antigen epitope complexes presented on cancer cells, such that it can stain T cells that are able to recognize Adpgk-presenting cancer cells (Fig. 7a, Supplementary Fig. 23). Control CpG + Adpgk elicited only 1.1% ASMTNEMLM-specific CD8<sup>+</sup> T cells among all live (DAPI<sup>-</sup>) CD8<sup>+</sup> cells. In contrast, iDR-NC/Adpgk induced 9.5% ASMTNEMLM-specific CD8<sup>+</sup> T cells (Fig. 7b, c), an 8-fold increase relative to CpG+Adpgk. Moreover, these ASMTNEMLM-specific CD8<sup>+</sup> T cells in nanovaccine-immunized mice upregulated the expression of programmed death receptor 1 (PD-1) (Fig. 7d), an immune checkpoint whose expression on T cells, especially antigen-specific CD8<sup>+</sup> T cells, can be upregulated by immunostimulation43. Thus, the upregulated PD-1 expression on ASMTNEMLM-specific CD8<sup>+</sup> T cells further implied potent immunostimulation of iDR-NC/Adpgk. Remarkably, on day 49, mice immunized with iDR-NC/Adpgk showed large fractions of central memory CD8<sup>+</sup> T cells (CD8<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup>) (Fig. 7e), indicating durable T cell responses. Again, the potent and durable immunity was attributed to the potent synergistic immunostimulation of iDR-NCs as well as the co-delivery of Adpgk with iDR-NCs via iDR-NCs/Adpgk complexes.

iDR-NC/neoantigen nanovaccines for cancer immunotherapy. Motivated by the potent and durable T cell responses induced by iDR-NC/neoantigen complexes, we then studied tumor immunotherapy using the above nanovaccines. In addition to durable immunity, another characteristic of immunotherapy is systemic efficacy. We studied neoantigen-specific immunotherapy of MC38 tumor in syngeneic C57BL/6 mice. MC38 cells (1 x 10<sup>5</sup>) were i.v. injected into C57BL/6 mice. On day 10, mice were treated with iDR-NC/Adpgk complexes (2 nmol CpG equivalents, 17 μg Adpgk), followed by boosting on day 16 and day 22. At the end of the study (day 40), the metabolic activity of metastatic-like tumors in lungs was determined using 18F-fludeoxyglucose (FDG), a metabolic radiotracer for tumor diagnosis. At 1 h post FDG injection, tumor burden in lung from randomly picked PBS-treated mice were clearly illuminated in PET-CT and showed apparently higher FDG signal intensities than background muscle.
Discussion

Nanovaccines are an important class of nanomedicines that hold substantial potential for disease treatment including cancer immunotherapy. Nucleic acid nanovaccines are particularly attractive because (1) the structural programmability of nucleic acids enables precise engineering of the nanostructure morphology and pinpoint loading of theranostic agents, and (2) nucleic acids have intrinsic immunomodulatory functionalities (e.g., immunostimulatory CpG and poly(I:C)). These characteristics make nucleic acid nanovaccines especially appealing for combination cancer immunotherapy, for which nanovaccines that can co-deliver DNA, RNA, and peptide therapeutics are of significant interest. For example, immunomodulatory DNA and RNA adjuvants can exploit independent signaling pathways to synergistically elicit antitumor immunity, and combination of these nucleic acid adjuvants with peptide antigens can elicit potent and durable T cell responses, which is critical for efficacious cancer immunotherapy.

In this study, we first developed nucleic acid nanomaterials, termed iDR-NCs, that were self-assembled from both DNA and RNA in a two-in-one reaction system; we then constructed immunotherapeutic nanovaccines by loading tumor neoantigens into iDR-NCs. Specifically, we optimized RCR/RCT reactions in the same solution to concurrently generate tandem DNA and RNA, which were further self-assembled into intertwining DNA-RNA MFs. While separate RCR or RCT have been previously employed to construct DNA or RNA nanostructures, iDR-NCs represent the first hybrid DNA-RNA nanostructures that were generated via combined RCR and RCT in the same reaction system. The combined RCR and RCT essentially incorporated both DNA and RNA therapeutics into the single nanomedicine. Specifically, we engineered MFs to be assembled of synergistic CpG and Stat3-silencing shRNA, such that TLR9 and STAT3 signaling pathways were synergistically leveraged to elicit potent immunostimulation in APCs for cancer immunotherapy. To improve delivery to LNs and LN APCs while maintaining the biosafety of nanovaccines, we synthesized a biocompatible PPT-g-PEG copolymer to shrink MFs into NCs. Although polyethyleneimine (PEI) may also shrink this type of microstructures, the cytotoxicity of highly cationic PEI raises serious safety concerns. To address this issue, we synthesized biocompatible PEG-grafted polypeptides (PPT-g-PEG) copolymers to shrink MFs. Further, given the hydrophobicity of PPTs in this copolymer, we physically loaded tumor-specific neoantigens into iDR-NCs via hydrophobic interactions between neoantigens and PPTs. Most neoantigens are hydrophobic, thus loading neoantigens into iDR-NCs by hydrophobic interactions is expected to be widely applicable to most neoantigens. Furthermore, hydrophilic...
neoantigens could be loaded into iDR-NCs by chemically conjugating neoantigens with PPT-g-PEG. Moreover, this multifunctional PPT-g-PEG is expected to shed PEG in the acidic endolysosome of APCs to enhance the proton sponge effect of cationic PPTs and then facilitate cytosolic delivery of shRNA and peptide neoantigens. Using quantitative PET imaging, super-resolution fluorescence imaging and flow cytometry, we demonstrated that iDR-NC/neoantigen nanovaccines were efficiently delivered to LNs and LN APCs. We further showed that CpG/shRNA-Stat3 iDR-NCs synergistically activated APCs in vitro. Although the synergistic efficacy between CpG and shRNA-Stat3 for in vivo T cell responses or tumor immunotherapy has yet to be investigated, the potent T cell responses and tumor therapeutic efficacy of iDR-NC/Adpgk are presumably attributed, at least in part, to this synergistic APC activation.

Potent and durable neoantigen-specific antitumor T cell responses were elicited by iDR-NC/Adpgk nanovaccines. Neoantigens and neoantigen-specific CTLs have been preclinically and clinically shown to be pivotal in tumor immunotherapy⁷⁵. However, natural neoantigen-specific CTLs are extremely rare (e.g., < 0.001% in colorectal cancer⁴⁷,⁴⁸), likely due to low clonal neoantigen burden, inefficient antigen processing and cross presentation, as well as immunosuppression. Delivering exogenous neoantigens can enhance the frequencies of neoantigen-specific CTLs to improve cancer immunotherapy. Therefore, nanovaccines co-delivering synergetic adjuvants and neoantigens are promising to potentiate neoantigen-specific immunity for tumor immunotherapy.⁷⁵,⁴⁴,⁴⁶,⁴⁷

Taken together, iDR-NCs represent a general technology to construct hybrid DNA-RNA nanostructures that can be further loaded with hydrophobic peptide therapeutics. Specifically, in this study, CpG and Stat3 shRNA were used as building blocks, with PPT-g-PEG copolymers as shrinkage materials. The resulting iDR-NCs were further loaded with tumor neoantigens and served as potent synergetic nanovaccines for personalized combination cancer immunotherapy.

Methods
DNA synthesis. DNA was synthesized using reagents from Glen Research (Sterling, VA) and Chemgenes (Wilmington, MA), on an Applied Biosystems ABI 392 DNA synthesizer. DNA was deprotected according to manufacturer’s instructions, followed by purification on a RP-HPLC (Dionex Ultimate 3000, ThermoFisher Scientific, Waltham, MA). Dye-labeled DNAs were purchased from IDT (Coralville, IA).

Synthesis of PPT-g-PEG. Synthesis of γ-propargyl-L-glutamate hydrochloride: L-glutamic acid (6.0 g, 40.8 mmol) was suspended in 200 mL propargyl alcohol. 14.3 mL of chlorotrimethylsilane was added into the suspension dropwise over 2 h at room temperature, followed by further stirring for 2 days. The reaction mixture was purified by precipitation into diethyl ether, and further purified by three-times precipitation from methanol (50 mL) to diethyl ether (0.2 L). The product was filtrated and dried (4.71 g, yield: 52 %). 1H NMR (300 MHz, CD3OD, ppm) 8: 4.73 (δ, 2 H), 4.44 (ddd, 2.11, 2.5 Hz, 1 H), 4.05 (t, 2.5 Hz, 2 H), 2.65 (m, 2 H), 2.39 (m, 2 H), 2.11 (m, 2 H).

Synthesis of γ-propargyl-L-glutamate hydrochloride: γ-propargyl-L-glutamate hydrochloride (2.0 g, 9.0 mmol) and triphosgene (2.7 g, 3 mmol) were suspended in 100 mL ethyl acetate, and were then stirred under reflux for 3 h until the reaction mixture became clear. The crude product was extracted with 100 mL ethyl acetate, and then were stirred under reflux for 3 h at room temperature, followed by further stirring for 2 days. The reaction mixture was purified by precipitation into diethyl ether, and further purified by three-times precipitation from methanol (50 mL) to diethyl ether (0.2 L). The product was filtrated and dried (4.71 g, yield: 52 %). 1H NMR (300 MHz, CDCl3, ppm): δ 7.47 (d, J = 2.5 Hz, 1 H), 2.95 (t, J = 2.5 Hz, 1 H), 2.65 (m, 2 H), 2.39–2.11 (m, 2 H).
Hydrophobic antigens with PPT moieties in iDR-NCs. After incubation in PBS, the supernatant was removed, followed by washing twice using PBS.

In a typical experiment, 2.5 mg/mL copolymer in PBS was mixed with MFs on a vortex for 10 min. The reaction mixture was then bubbled by nitrogen flow for 10 min, and placed under UV light with a wavelength of 365 nm for 2.5 h. The mixture was transferred into a presoaked dialysis membrane tubing (MWCO 6–8 kDa), dialyzed against nanopure water for 2 days, and then lyophilized to get the final product (568 mg, yield: 86%).

Physical characterization of iDR-NCs

Self-assembly of iDR-NCs and iDR-NC/antigen complexes

Hybrid DNA-RNA templates were circularized using ligation helper DNA (from Dr. Robert A. Seder Lab at NIAID) were cultured in DMEM medium with L-glutamine. DC2.4 cells (Dr. Jonathan W. Yewdell Lab at NIAID) were cultured in RPMI medium with L-glutamine with L-glutamine. A. Seder Lab at NIAID) were cultured in DMEM medium with L-glutamine with L-glutamine.

Super-resolution imaging on instant SIM

DNA interference. siRNA or control were transfected using Lipofectamine2000 (Thermo Fisher Scientific) following manufacturer’s guidance. Stau3 siRNA, pri-mers for Stau3 mRNA, Stattic, and p-STAT3 antibody were purchased from Santa Cruz Biotechnology.

Concentrations of proinflammatory factors

In vitro cell uptake of iDR-NCs

In vitro cell uptake was first studied using confocal laser scanning microscopy and flow cytometry. FITC-labeled AluCpG was incubated with RAW264.7 cells or DC2.4 cells, and stained with LysoTracker Red DND-99 (Life Technologies, Carlsbad, CA) and Hoechst 33342 (Life Technologies). Cells were incubated with RAW264.7 cells or DC2.4 cells, and stained with LysoTracker Red DND-99 (Life Technologies, Carlsbad, CA) and Hoechst 33342 (Life Technologies).

Expression levels of costimulatory factors on APCs

RAW264.7 macrophages and DC2.4 cells were studied by flow cytometry for the expression levels of costimulatory factor CD80. Cells were seeded into 24-well plate, and one day later treated with iDR-NCs or the corresponding control regimens at the specified concentration.

Estimation of nucleic acid yields of RCT/RCT

iDR-NCs were treated with 5 mM EDTA, which chelated Mg2+ and dissolved ssDNA. ssDNA was purified by removing EDTA, Mg2+, and PDI using centrifugation filtering (Millipore, Billerica, MA). The absorbance (260 nm) of the resulting DNA/RNA was determined on a Genesy 10 UV-Vis spectrometer (ThermoFisher Scientific, Waltham, MA) and converted to the equivalent of CpG or shRNA.

Biodistribution of iDR-NCs

iDR-NCs were treated with 5 U/mL DNase I (New England Biolabs, Ipswich, MA) at 37 °C for 1 h, followed by nuclease deactivation at 75 °C for 1 h. The stability of iDR-NCs against thermal denaturation was performed by heating iDR-NCs at specified temperature for 1 h. The morphology of the above treated iDR-NCs was examined using SEM.

Cell culture

RAW264.7 macrophages (ATCC) and MC38 cells (from Dr. Robert A. Seder Lab at NIAID) were cultured in DMEM medium with L-glutamine. DC2.4 cells (Dr. Jonathan W. Yewdell Lab at NIAID) were cultured in RPMI medium with L-glutamine. A. Seder Lab at NIAID) were cultured in DMEM medium with L-glutamine. DC2.4 cells (Dr. Jonathan W. Yewdell Lab at NIAID) were cultured in RPMI medium with L-glutamine.

Concentrations of proinflammatory factors

Proinflammatory factors (TNFα, IL-6, IL-12p40) in cultured RAW264.7 or DC2.4 cells were quantified using ELISA according to manufacturers’ instructions. Cell culture medium from cells that were collected at the specified time points post treatment was added to the corresponding control regimens. Media was diluted according to manufacturers’ instructions. Cell culture medium was then collected and centrifuged to remove any debris. The concentrations of cytokines in the culture medium were determined by ELISA (Life Technologies, Carlsbad, CA) as per manufacturer’s instructions.

Expression levels of costimulatory factors on APCs

RAW264.7 macrophages and DC2.4 cells were studied by flow cytometry for the expression levels of costimulatory factor CD80. Cells were seeded into 24-well plate, and one day later treated with iDR-NCs or the corresponding control regimens at the specified concentration.
Animal studies. All animal work was conducted in accordance with the NIH Guide for the Care and Use of Animals under protocols approved by the NIH Clinical Center Animal Care and Use Committee. All studies on animals were evaluated in a blinded manner to investigators without prior knowledge of the specific treatments.

In vivo delivery of iDR-NC/antigen into LN lymphocytes. Alexa 555 was labeled on iDR-NCs through an Alexa 555-conjugated primer. CSINFEK1 model antigen was modified with an FITC to be CSINFEK1-FITC-L. iDR-NC-Alexa 555/CSINFEK1-L (in 50 μL reaction solution) were s.c.-injected into C57BL/6 mice at the tail base, and one day later, LN cells were collected and stained, treated with collagenase D (1 mg/mL Sigma) and DNase I (10 μU/mL NEB) for 2 h at 37 °C to prepare single cells. Cells were filtered through a 40-μm strainer to remove tissue debris. The resulting single cells were stained with B220 (anti-B220-Alexa647, clone RA3-6B2, Biolegend) for B cells, F4/80 (anti-F4/80-APC, clone BM8, eBioscience) for macrophages, and CD11c (anti-CD11c-APC, clone N418, Biolegend) for DCs. Flow cytometry was conducted on a BD LSRFortessa X-50 flow cytometer. Results were analyzed in macrophage populations using FlowJo Software (TreeStar, Ashland, OR).

Immune memory analysis. Mice were vaccinated as described above. Peripheral blood was collected to analyze antigen-specific CD8+ T cells and memory T cells. Immune memory was analyzed by flow cytometric analysis of CD62L and CD44. Briefly, red blood cells were lysed, and blood cells were then collected, washed with PBS buffer, blocked with anti-CD62L/CD43 for 10 min at room temperature. Blood clots were removed using a plate filter. Cells were washed twice in PBS and cells were stained with DAPI in PBS buffer supplemented with anti-CD16/CD32 for 10 min at room temperature. Next, cells were added with dye-labeled staining cocktail including anti-CD8-APC-Cy7 (BioLegend), Dextramer-PE (Immudex), anti-PE-1BV42 (BioLegend), according to manufacturer’s instructions. Cells were stained at room temperature for 30 min, washed, and 100 μL Cytofix was added into each well to resuspend cells for 4°C for 20 min. Cells were then washed with Perm/Wash buffer, and resuspended for flow cytometric analysis on a BD LSRFortessa X-50 flow cytometer. Data were analyzed using FlowJo V10 and GraphPad Prism 4. (La Jolla, CA).

Tumor model and combination cancer immunotherapy. C57BL/6 mice (6–8 weeks) were i.v. injected with MC38 cells (1 × 106). On day 10, mice were randomly divided into groups (6–7 per group) that were respectively vaccinated with (1) PBS, (2) CpG and Adpgk, and (3) iDR-NCs and Adpgk, by subcutaneous injection of 50 μL. On day 10, 16, and day 22 post inoculation. Tumor burden was quantified at the end of study using FGD radio-tracer. Specifically, mice were anesthetized for 30 min using isoflurane/O2 (2% v/v) before injection. Anesthetized mice were injected i.p. with FGD (3.7 MBq per mouse) in PBS (100 μL). Mice continued to be anesthetized for 1 h, and then, one mouse from each group was randomly picked to be scanned for PET/CT on a nanoScan PET/CT scanner (Mediso Medical Imaging Systems). Meanwhile, mice were euthanized and organs of interest were resected, followed by measuring 18F radioactivity on a gamma-counter (Wallac Wizard 1480, PerkinElmer). The radioactivity in organs was converted to calculate the percentages of the %ID and %ID per g of organs of interest. Results were analyzed using GraphPad Prism 4 (La Jolla, CA).

Data availability. The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding authors on request.

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Author contributions

G.Z. and F.Z. conceived and designed the project; G.Z., L.M., O.J., Z.W., Y.L., X.F. and F.Z. performed the experiments; G.Z., L.M., X.F., A.J., H.D.V., G.N., F.Z., Q.W., H.S. and X.C. analyzed data; G.Z., L.M., H.D.V., B.C.Y., F.Z., H.S. and X.C. co-wrote the paper; X. C. supervised all studies. All the authors have approved the final version.

Additional information

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