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Multifunctional Hybrid Nanoconjugates for Efficient In Vivo Delivery of Immunomodulating Oligonucleotides and Enhanced Antitumor Immunity**

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The design and synthesis of multifunctional nanomaterials have provided potential applications in biomedical fields such as molecular imaging and drug delivery.[1] Recently, bio-derived and/or synthetic nanostructured materials capable of modulating the immune system have become of interest in immunology-related fields of nanomedicine.[2] Dendritic cells (DCs) represent important targets for immunotherapeutics in cancer because they can capture tumor antigens that are released from tumor cells and migrate to the tumor-draining lymph nodes, where they present the antigens to T cells and secrete the pro-inflammatory cytokines that enhance T cell activation (Figure 1 a).[3] This results in tumor-antigen-specific activation of T cells that contributes to tumor rejection.[3] However, because cancer cells can prevent the maturation and function of DCs by a variety of mechanisms, DCs that infiltrate into tumor microenvironments usually exhibit an immature phenotype.[3a,4] Whereas mature DCs can induce potent antigen-specific antitumor immunity, immature DCs do not promote T cell responsiveness and instead induce infiltration of regulatory T cells.[3a,4b,5] Therefore, it is necessary that DCs in tumor microenvironments are matured with immunostimulatory factors such as inflammatory cytokines, Toll-like receptor (TLR) ligands, and CD40 ligands.[6] As an immunostimulatory TLR agonist, TLR9-specific unmethylated cytosine–guanosine (CpG) oligodeoxynucleotides (ODNs) are already in clinical trials for melanoma.[7] Unmethylated CpG ODNs bind to TLR9 and are efficiently internalized by various antigen-presenting cells, which can initiate a cascade of innate and adaptive immune responses.[6,8] However, immunosuppressive factors, such as signal transducer and activator of transcription-3 (STAT3), represent a major limitation for DC-based cancer therapies.[4b,9] Activation of STAT3 in DCs inhibits the expression of numerous immunostimulatory molecules triggered by TLR ligands that regulate T cell activation (Supporting Information, Figure S1).[10]

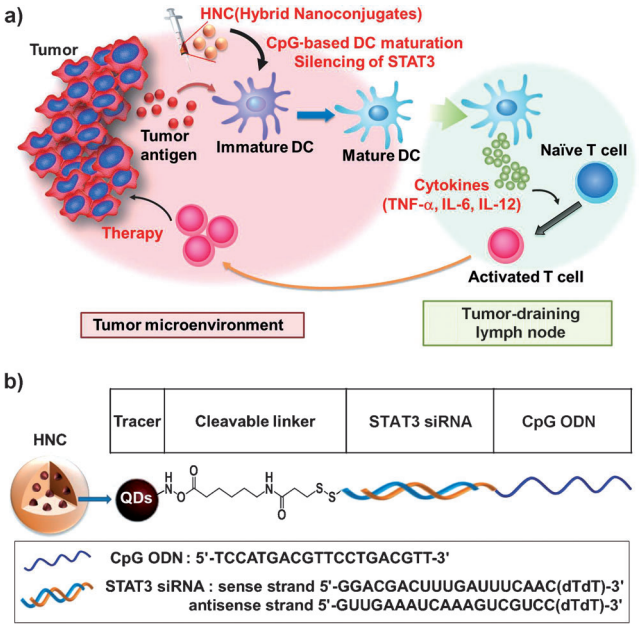

Figure 1. a) Schematic illustration of HNC-based delivery of immunomodulating oligonucleotides to DCs within a tumor microenvironment for the silencing of immunosuppressive genes (STAT3 siRNA) and the activation of TLRs (CpG ODNs), leading to therapeutic antitumor immune responses. b) Scheme of the composition of HNCs based on polymer nanoparticles containing QDs (as imaging tracers) conjugated to CpG ODNs and STAT3 siRNAs using a cleavable disulfide linker.

Because STAT3 influences the DC maturation process mediated by CpG ODNs, we reasoned that the simultaneous silencing of STAT3 by small interfering RNAs (siRNAs) and activation of TLR9 by CpG ODNs might effectively induce antitumor immunity in the tumor microenvironment (Figure 1 a and Figure S1).[10b] Thus, we focused on the development of an immunomodulatory nanodelivery system for the simultaneous in vivo delivery of CpG ODNs as a DC activator and STAT3 siRNAs as a silencer of immunosuppressive genes in the DCs of tumor microenvironments (Figure 1 b). Although the ability of siRNAs to control the expression of specific genes makes them an attractive new class of drugs, with broad potential for the treatment of

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diverse human diseases,[11] most of the efforts to develop siRNA-based anticancer therapeutics have focused on delivery of siRNAs that can directly silence specific genes in cancer cells.

Herein, we have designed and synthesized an immunomodulatory hybrid nanoconjugate (HNC) system based on polymer nanocomposites containing quantum dots (QDs) that are conjugated with STAT3 siRNAs and CpG ODNs (Figure 1b). Figure 1b shows the strategy for the synthesis of the poly(lactic-*co*-glycolic acid) (PLGA) nanocomposites containing QDs conjugated to immunomodulating oligonucleotides (CpG ODNs and STAT3 siRNAs) by a cleavable disulfide linker (see also Figure S2). We designed the nanodelivery system, HNCs, for the following purposes: 1) creating a single molecule (CpG-STAT3 siRNA) capable of simultaneously delivering CpG ODN and siRNA to DCs in vivo by linking the two oligonucleotides; 2) increasing the encapsulation efficiency of hydrophilic small CpG ODNs and siRNA molecules into a hydrophobic PLGA matrix by conjugation with QDs (Table S1); 3) allowing for stimuli-responsive cleavage and release of CpG ODNs and STAT3 siRNAs into the cells by introducing a cleavable disulfide linker (S–S) between the QDs and the oligonucleotides;[12] 4) assessing the delivery of siRNAs and CpG ODNs into DCs and the in vivo trafficking of activated DCs to lymph nodes by near-infrared (NIR) fluorescence imaging by using NIR-emitting QDs;[13] 5) enhancing the uptake and controlling the release of siRNAs and CpG ODNs into DCs in vivo by encapsulating the QD-biomolecule conjugates with PLGA (Figure S3).[3c,d,14]

Amine-modified QDs were conjugated to thiol-containing CpG ODNs, STAT3 siRNA, and linked CpG-STAT3 siRNA using succinimidyl 3-(2-dipyridyldithio)propionate (SPDP) cross-linkers because the disulfide moiety (S–S) between the QDs and the oligonucleotides could be used to release them when the QDs encounter the acidic conditions of the endosomes upon delivery into cells (Figure S2).[12,15] Next, the QD-CpG ODN, QD-STAT3 siRNA, and QD-CpG-STAT3 siRNA conjugates were encapsulated into a PLGA matrix using the double-emulsion solvent-evaporation method.[3c,d] The physical parameters and doses of oligonucleotides in each HNC are summarized in Table S2. The average diameters of the HNCs conjugated with either CpG ODNs (HNC_{CpG}), STAT3 siRNAs (HNC_{STAT3}), or both ($HNC_{CpG/STAT3}$) were 159.1 ± 27.3 nm, 136.8 ± 27.0 nm, 154.5 ± 21.3 nm, and the surface charges were −28.50 mV, −29.57 mV, −30.57 mV, respectively (Table S2). The scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of the $HNC_{CpG/STAT3}$ also revealed that the QDs were randomly distributed in the PLGA nanoparticle matrix (Figure 2a). When the $HNC_{CpG\text{-}STAT3}$ solutions were illuminated with light ($\lambda_{ex} = 670$ nm), they generated strong NIR signals ($\lambda_{em} = 800$ nm), whereas no signal was detected in the buffer alone (PBS) and nanoconjugates without QDs (HNC_{empty}; Figure 2b). As the concentration of $HNC_{CpG/STAT3}$ increased, their cellular uptake into bone-marrow-derived DCs (BMDCs) also increased (Figure 2c and Figure S4). When BMDCs were treated with various concentrations (from 100–500 nM) of $HNC_{CpG/STAT3}$ for 24, 48, or 72 hours, no cytotoxic effects were detected, regardless of concentration (Figure S5).

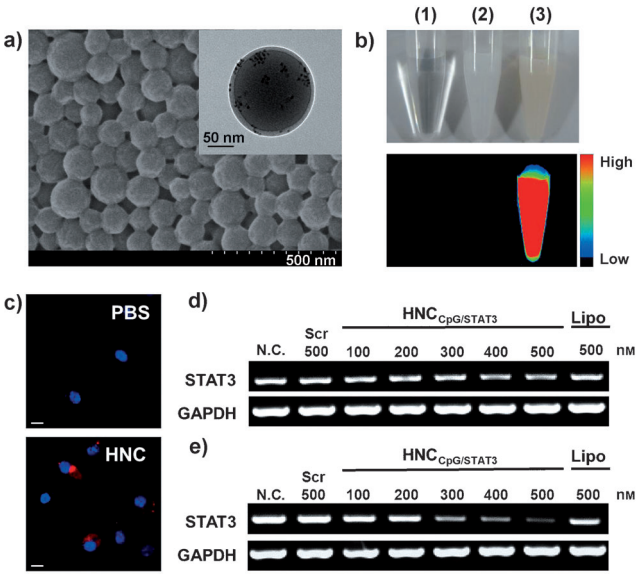

Figure 2. Characterization and functional analysis of HNCs. a) SEM and TEM (insert) images of $HNC_{CpG/STAT3}$. b) In vitro optical and NIR fluorescence images (red) of (1) PBS, (2) HNC_{empty}, and (3) $HNC_{CpG/STAT3}$. c) NIR fluorescence microscopy images of DCs internalized by $HNC_{CpG/STAT3}$ (red). The nuclei were stained with Hoechst 33342 (blue). Scale bars = 10 μm. d,e) In vitro gene-silencing effect of $HNC_{CpG/STAT3}$ on STAT3 mRNA levels in DCs. mRNA levels were measured by RT-PCR after 24 h (d) and 48 h (e) treatment. Lipo indicates Lipofectamine transfection. N.C. = negative control; Scr = scrambled siRNA.

In vitro gene-silencing effects of the $HNC_{CpG/STAT3}$ were determined by RT-PCR analysis of the STAT3 mRNA levels (Figure 2d,e). STAT3 mRNA expression was slightly

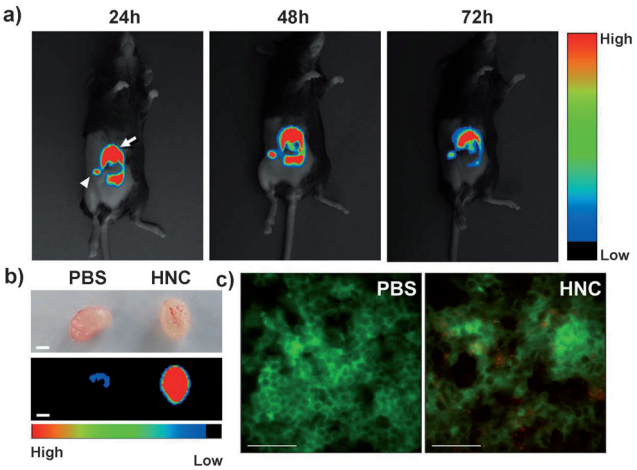

Figure 3. a) In vivo NIR images of an tumor-draining inguinal lymph node at 24 h up to 72 h after intratumoral injection of $HNC_{CpG/STAT3}$ (Arrow: tumor region, Arrowhead: inguinal lymph node). b) Ex vivo optical (top) and NIR (bottom) images of a dissected inguinal lymph node. Scale bars = 1 mm. c) Immunofluorescence analysis of a dissected inguinal lymph node. Slides were stained with FITC-labeled anti-CD3e antibody (green; T cell marker). DCs were labeled with $HNC_{CpG/STAT3}$ (red). Scale bars = 90 μm.

decreased at 24 hours after transfection with 100–500 nM siRNA (Figure 2d). In contrast, the extent of STAT3 knockdown 48 hours after transfection (86% at 500 nM) was higher than at 24 hours (32% at 500 nM) because of the controlled-release properties of PLGA (Figure 2e). The gene-silencing efficiency of $HNC_{CpG/STAT3}$ was even higher than that mediated by the commercialized transfection agent, Lipofectamine (Lipo; 40% at 500 nM, 48 h). Inspired by these gene-silencing effects in vitro, we next evaluated the in vivo gene silencing and antitumor immune responses of the three HNCs administered by intratumoral injection into mice. Tumor-infiltrating DCs are highly phagocytic and are efficient at taking up oligonucleotides, because of their immature phenotype.[3a] To assess the delivery of the injected $HNC_{CpG/STAT3}$, the NIR signals from the QDs were detected in the inguinal lymph nodes from 24 to 72 hours (Figure 3a). NIR fluorescence was detected in the inguinal lymph nodes starting at 24 hours and the signal intensity increased continuously up to 48 hours. However, the NIR signal intensity in the inguinal lymph nodes began to decrease after 72 hours (Figure 3a). To investigate the localization of DCs in the lymph node, the dissected inguinal lymph nodes were analyzed by immunofluorescence (Figure 3b,c). The co-localized signals from the T cells (green) and the DCs labeled with $HNC_{CpG/STAT3}$ (red) suggested that the DCs in the tumor microenvironment successfully migrated into T cells, residing in tumor-draining inguinal lymph nodes as indicated by the NIR macroscopic imaging (Figure 3c, Figure S6).

Intratumoral injections of $HNC_{CpG/STAT3}$ also resulted in effective gene silencing in DCs within the inguinal lymph nodes, compared to the control, PBS (Figure 4a). STAT3 mRNA expression was significantly decreased after 48 hours treatment with $HNC_{CpG/STAT3}$ (73% silencing) and HNC_{STAT3} (64% silencing; Figure 4a). A wide variety of cytokines can be expressed by mature DCs, including TNF-α, IL-6, and IL-12.[16] As the loss of STAT3 in DCs has been shown to upregulate the expression of Th1 cytokines, which can be greatly amplified by CpG ODNs, we assessed the change in Th1 cytokine levels in the DCs. To evaluate the role of the HNC in immune modulation, we analyzed immunostimulatory Th1 cytokines (TNF-α, IL-6, and IL-12) in DCs isolated from the inguinal lymph nodes of ten mice. Both the real-time PCR and RT-PCR results revealed that cytokine expression was increased when the DCs were treated with HNC_{CpG} (TNF-α: 2.78 times, IL-6: 2.38 times, IL-12: 1.94 times) and $HNC_{CpG/STAT3}$ (TNF-α: 3.97 times, IL-6: 2.70 times, IL-12: 3.28 times; Figure 4b–e), compared to the control, PBS. Treatment with the $HNC_{CpG/STAT3}$ more efficiently increased TNF-α, IL-6, and IL-12 cytokine secretion in DCs compared to HNC_{CpG} and HNC_{STAT3}, suggesting that this immune response was synergistically elicited by both CpG ODN-based immunostimulation and gene silencing of immunosuppressive STAT3 by siRNA. To evaluate the in vivo antitumor effects of HNC, we intratumorally injected HNCs into B16F10 tumor-bearing mice, five times at two-day intervals. As shown in Figure 4f, injection of $HNC_{CpG/STAT3}$ led to a statistically significant inhibition of tumor growth that lasted for the entire observation period of 20 days, compared to the other three groups (PBS, HNC_{CpG}, HNC_{STAT3}). Taken together, simultaneous in vivo delivery of STAT3 siRNAs and CpG ODNs by HNCs induced both the inhibition of STAT3 and activation of DCs by CpG ODNs, giving synergistic antitumor effects.

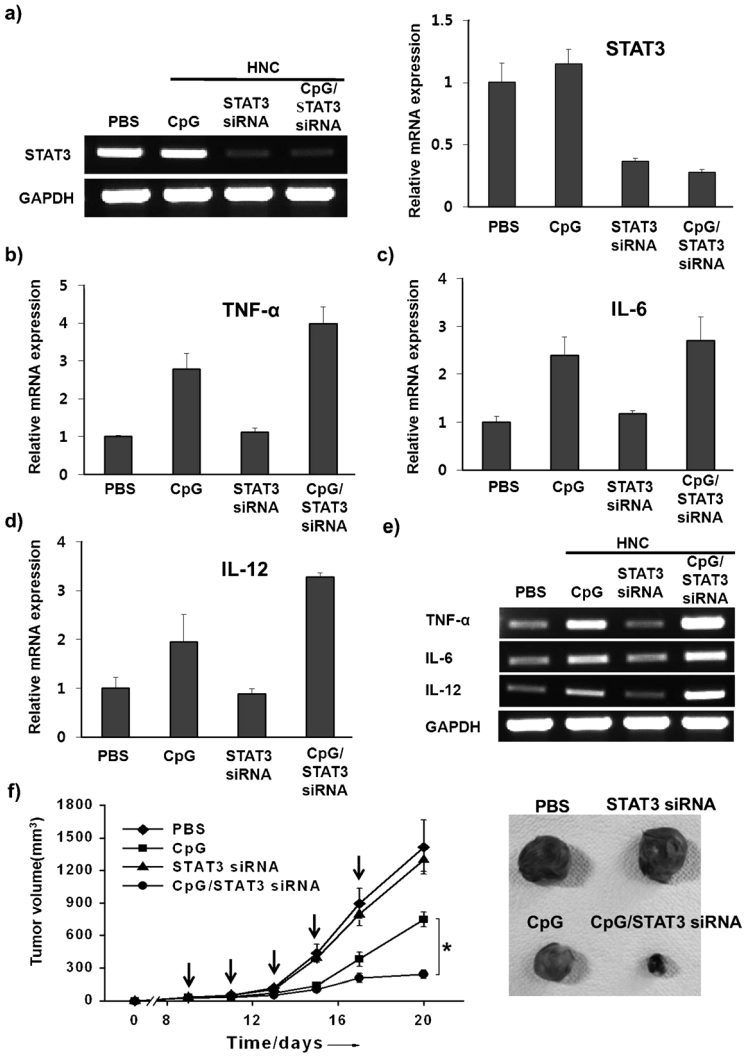

Figure 4. a) In vivo gene-silencing effects of HNCs on STAT3 mRNA levels in DCs. The mRNA levels were measured by RT-PCR (left) and real-time PCR (right) after 48 h treatment. b–e) In vivo immunostimulatory cytokine gene expression in DCs of a tumor-draining inguinal lymph node. mRNA levels were analyzed by real-time PCR (b–d) and RT-PCR (e). Data are the average of three experiments with the corresponding standard deviation values. f) Time-course of tumor growth ($n = 7$) after five injections (black arrow) of either PBS, HNC_{CpG}, HNC_{STAT3}, or $HNC_{CpG/STAT3}$ (left). $* = p < 0.001$, paired t-test. Photographs of the tumor tissue from each group are also shown (right).

In summary, we have designed and synthesized immunomodulatory hybrid nanoconjugates (HNCs) based on polymer nanocomposites containing QDs (as imaging tracers) conjugated with CpG ODNs (as a TLR9 ligand) and STAT3

siRNAs (as an immunosuppressive gene silencer). These HNCs efficiently targeted immune cells, induced TLR activation, and silenced immunosuppressive genes. Simultaneous delivery of STAT3 siRNAs and CpG ODNs to the tumor microenvironment caused the inhibition of STAT3 along with activation of DCs by CpG ODNs, and their antitumor effects were found to be synergistic. By using NIR-emitting QDs, the migration of in vivo DCs to lymph nodes was tracked by real-time NIR fluorescence imaging. For future clinical applications, other types of non-toxic materials (such as non-cadmium based QDs and iron oxide nanoparticles) could be an alternative to the QDs used herein. Although the design of materials capable of modulating the immune system is still an emerging field,[2] multifunctional HNCs represent a potentially useful nanotechnology platform for enhanced antitumor immunity, as well as molecular imaging probes for DC-based cancer therapy.[1,3] In the future, these studies could be used to develop types of immune-cell-based cancer therapy.

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