Multivalent Polymer Nanocomplex

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Multivalent Polymer Nanocomplex Targeting Endosomal Receptor of Immune Cells for Enhanced Antitumor and Systemic Memory Response**

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Abstract: *We have designed and synthesized linear polymer-based nanoconjugates and nanocomplexes bearing multivalent immunostimulatory ligands and also demonstrated that the synthetic multivalent nanocomplexes led to an enhanced stimulation of immune cells in vitro and antitumor and systemic immune memory response in vivo. We have developed hyaluronic acid (HA)-based multivalent nanoconjugates and nanocomplexes for enhanced immunostimulation through the combination of multivalent immune adjuvants with CpG ODNs (as a TLR9 ligand) and cationic poly(L-lysine) (PLL; for the enhancement of cellular uptake). The multivalent HA-CpG nanoconjugate efficiently stimulated the antigen-presenting cells and the multivalent PLL/HA-CpG nanocomplex also led to an enhanced cellular uptake as well as continuous stimulation of endosomal TLR9. The mice vaccinated with dendritic cells treated with the multivalent nanocomplex exhibited tumor growth inhibition as well as a strong antitumor memory response.*

The display of multivalent epitopes composed of identical or nonidentical ones, is important in various biological processes, such as host–pathogen interactions, cell surface adhesion, and viral entry.[1] In this respect, the design and chemical synthesis of nanobiomaterials with an ordered and repetitive nanostructure have been of great interest for the treatment of infectious diseases as well as for cancer immunotherapy.[2] Because nanoparticles have the intrinsic advantage that identical epitopes can be attached to their surfaces, most previous studies have focused on the use of solid spherical nanoparticles with a multivalent targeting ligand for applications in cancer diagnosis and therapy.[3] In addition, the flexibility of the multivalent ligand is one of the most important parameters involved in enhancing the binding efficiency of these ligands. To increase the flexibility of the targeting ligand on the surface of nanoparticles, spacer molecules with various lengths have been introduced between the targeting ligands and the surface of the nanoparticles.[4] Although some researchers have also attempted to use virus membranes with a multivalent hemagglutinin structure as potent vaccine adjuvants to boost the immune response, the laborious preparation procedures and safety issues associated with these viral membranes limited their widespread use.[5]

In this work, we designed and synthesized linear-type flexible polymer-based multivalent nanoconjugates and nanocomplexes consisting of multivalent immune-stimulating epitopes for the effective immunomodulation of antigen-presenting cells such as dendritic cells and macrophages. DNA containing unmethylated cytosine-phosphate-guanine (CpG), which are characteristic of bacterial and viral DNA, induces cytokine production from mammalian immune cells through recognition by Toll-like receptor 9 (TLR9) and activates innate and acquired immune responses.[6] The unmethylated CpG oligodeoxynucleotides (ODNs) can be recognized by TLR9 and induce a powerful immune response through cell-signaling pathways including MyD88-dependent nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which can induce the secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-12.[6] Thus, CpG ODNs have been widely used as a promising therapeutic tool to stimulate the immune response through the activation of TLR9 for various applications such as infectious diseases, allergy treatment, and cancer therapy.[7] However, naked CpG ODNs cannot penetrate efficiently through the cell membrane and are prone to rapid degradation by nucleases in the cytoplasm. Therefore, the efficient delivery of CpG ODNs into the target cell would be an effective strategy for ensuring their immunostimulatory activity. The advantages of intracellular delivery carriers for CpG ODNs are related to an increased cellular uptake, sustained release over a long time, and protection against degradation by nucleases. After cellular uptake, CpG ODNs can be recognized by TLR9 in the endosome. Endosomal TLR9 is the crucial site of interaction with CpG ODNs for increasing immune stimulation. Therefore, the CpG ODNs need to be retained in the endosome as long as possible, and the endosomal escape of CpG ODNs is undesirable. Thus, continuous stimulation of endosomal TLR9 would enhance the immunostimulatory activity of CpG ODNs.[8] Herein, we

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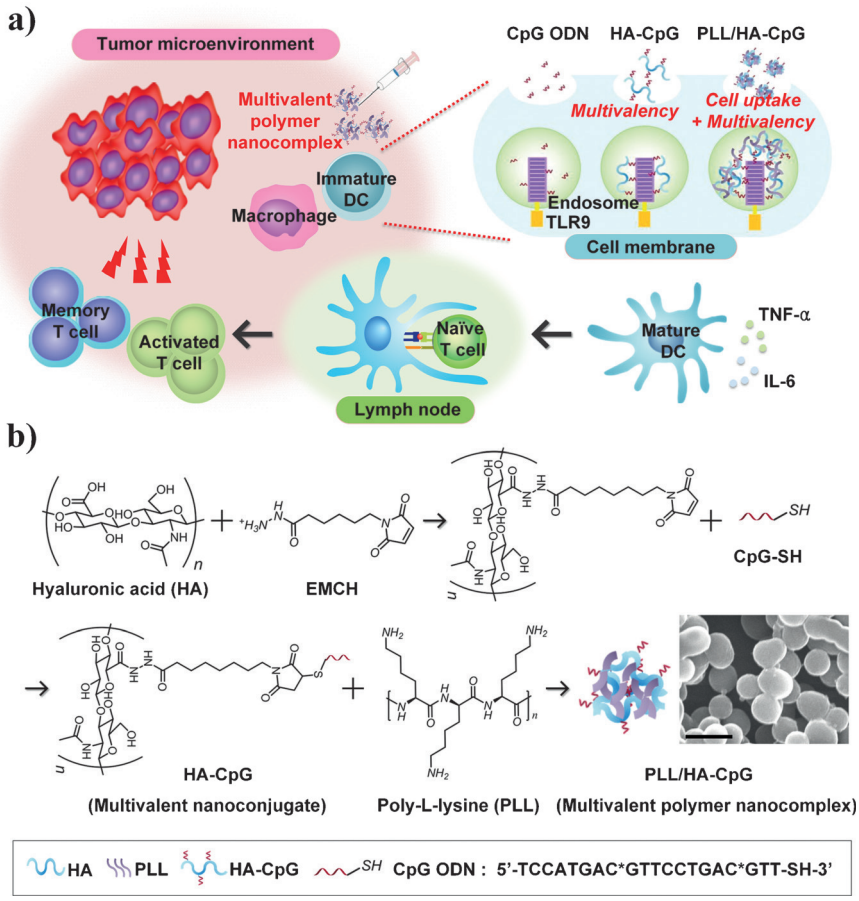

Scheme 1. a) Illustration of enhanced antitumor and memory response of the polymer nanocomplex with multivalent CpG ligands. After entering immune cells, the CpG ODNs can be recognized by Toll-like receptor 9 (TLR9) and induce a powerful immune response. The multivalent HA-CpG ligands are highly stimulatory TLR9 ligands that allow multivalent ligand–receptor interaction. The PLL/HA-CpG nanocomplexes synergistically elicit both enhanced cellular uptake (through the cationic PLL) and stimulation of TLR9 (by multivalent CpG ligands). b) Chemical scheme for the synthesis of multivalent HA-CpG conjugates and PLL/HA-CpG nanocomplexes. Scale bars = 1 μm.

focused on the development of a nanocomplex system for the effective intracellular delivery and subsequent multivalent display of CpG ODNs that can induce strong immunostimulatory activity through the continuous stimulation of TLR9 (Scheme 1 a). Hyaluronic acid (HA) is a biocompatible, biodegradable, nontoxic linear polysaccharide consisting of repeating units of D-glucuronic acid and *N*-acetyl glucosamine. HA has been used in the development of various drug-delivery and tissue-engineering systems.[9] The carboxylic acid and hydroxy groups on HA have been widely modified in conjugation reactions with drugs and proteins.[10,11] In our research, HA was adopted as a graft polymer to conjugate and deliver multivalent immunostimulatory ligands. HA-based multivalent nanoconjugates or nanocomplexes are expected to play an important role in receptor–ligand interactions involved in cellular recognition and signal processes of immune cells, because multivalent interactions are stronger than individual interactions and enhance the avidity of binding between interfaces and molecules. Scheme 1 a presents an illustration of the proposed model for the enhanced immunostimulatory effects induced by the multivalent nanoconjugates and nanocomplexes. After entering the cell, the multivalent HA-CpG conjugates can stimulate TLR9 through multivalent interaction and can increase the maturation of CpG-induced cells, and cytokine production by these cells is better than free CpG. To increase the cell-uptake efficiency of HA-CpG multivalent nanoconjugates, cationic poly-L-lysine (PLL) was used, which induced the formation of a PLL/HA-CpG ionic nanocomplex (Schemes 1 b and S1). In fact, several cationic polymers, such as polyethylenimine, polyamidoamine, and PLL, have been used for the delivery of genes into cells because they can easily form nanocomplexes with anionic DNA through electrostatic interactions under physiological conditions. Among these cationic polymers, PLL is known to remain in the endosome with little proton sponge effect because of its lack of amino groups.[12] Therefore, both the continuous stimulation of endosomal TLR9 by multivalent immune adjuvants (HA-CpG) and enhanced cellular uptake efficiency (through PLL) could be synergistically elicited by forming a PLL/HA-CpG nanocomplex.

The multivalent HA-CpG nanoconjugates were synthesized by conjugating the carboxylate group of HA and the thiol group of CpG ODNs with the trifluoroacetic acid salt of 3,3′-*N*-[ε-maleimidocaproic acid] hydrazide (EMCH; Scheme 1 b). The successful conjugation of EMCH to HA was confirmed by ^{1}H NMR and FTIR spectroscopy analyses (Figures S1 and S2).[13] The conjugation of CpG on HA-EMCH and the increase of molecular weight in the synthesized multivalent HA-CpG nanoconjugates were also characterized by ^{1}H NMR (Figure S3) and 13C NMR spectroscopy (Figure S4-a), size-exclusion chromatography (SEC) coupled with a multi-angle laser light-scattering (MALLS) detector(SEC-MALLS) (Figure S4-b), and UV spectrophotometry (Figure S4-c). The physical parameters and formulations of CpG ODNs in the PLL/HA-CpG nanocomplexes are also summarized in Table S1. The size and distribution of the PLL/HA-CpG nanocomplexes were measured using dynamic light scattering (Figure S5). The results indicate that the PLL/HA-CpG nanocomplexes exhibit a uniform size with a mean diameter of 536 nm and a surface charge of −36 mV (Table S1). It has been reported that NPs with sizes of approximately 500 nm are optimal for uptake into immune cells,[14] and the retention time of the NPs in the endosome was long.

To evaluate the cytotoxicity effect of multivalent HA-CpG nanoconjugates and PLL/HA-CpG nanocomplexes on bone-marrow-derived dendritic cells (BMDCs) and RAW264.7 cells, MTS assays were conducted in the presence of the indicated materials for 24 h and 48 h (Figure S6). No cytotoxicity effect on these cells was observed for either the HA-CpG nanoconjugates or PLL/HA-CpG nanocomplexes. We also examined the cellular internalization and uptake of the multivalent HA-CpG nanoconjugates and the PLL/HA-CpG nanocomplexes in BMDCs and RAW264.7 cells by fluorescence microscopy using CpG ODN labeled with the fluorophore FITC (Figure 1 a). Importantly, a bright green fluorescence signal was observed from the cytoplasm of the cells that were incubated with the multivalent PLL/HA-CpG nanocomplexes in contrast to those incubated with free CpG or multivalent HA-CpG nanoconjugates. We further examined the cellular uptake efficiency of the multivalent HA-CpG nanoconjugates and the PLL/HA-CpG nanocomplexes using flow cytometry analysis (Figures 1 b and S7). The relative fluorescence intensity revealed that the multivalent PLL/HA-CpG nanocomplexes exhibited 1.73- and 2.52-fold higher uptake efficiency than free CpG in BMDCs and RAW264.7 cells, respectively (Figure 1 b,c). However, the fluorescence intensities of the free CpG and multivalent HA-CpG nanoconjugates were similar. These results suggest that PLL enhanced the cellular uptake of the multivalent HA-CpG nanoconjugates.

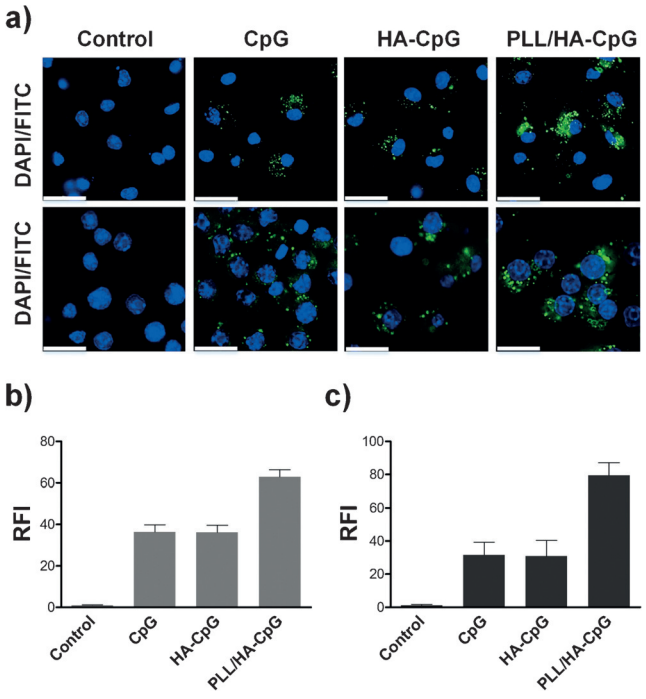

Figure 1. Cellular internalization and uptake of multivalent nanocomplexes. a) Fluorescence microscopy images of BMDCs (top) and RAW264.7 cells (bottom) incubated with 5 μg mL^{-1} of CpG concentration for 24 h at 37 °C. The nuclei were stained with Hoechst 33342 (DAPI, blue) and FTIC-labeled CpG-generated green fluorescence. Scale bars = 30 μm (top), and 60 μm (bottom). Cellular uptake efficiency was assessed by flow cytometry analysis of relative fluorescence intensity in b) BMDCs, and c) RAW264.7 cells (RFI = relative fluorescence intensity).

To assess the immunostimulatory activity, we measured the levels of secreted proinflammatory cytokines from immune cells treated by free CpG, multivalent HA-CpG nanoconjugates and PLL/HA-CpG nanocomplexes. IL-6 and TNF-α are important cytokines that are representative of the antitumor activity of BMDCs and RAW264.7 cells.[15] Whereas the control and HA exhibited no immunostimulatory activity, both the multivalent HA-CpG nanoconjugates and PLL/HA-CpG nanocomplexes highly stimulated the

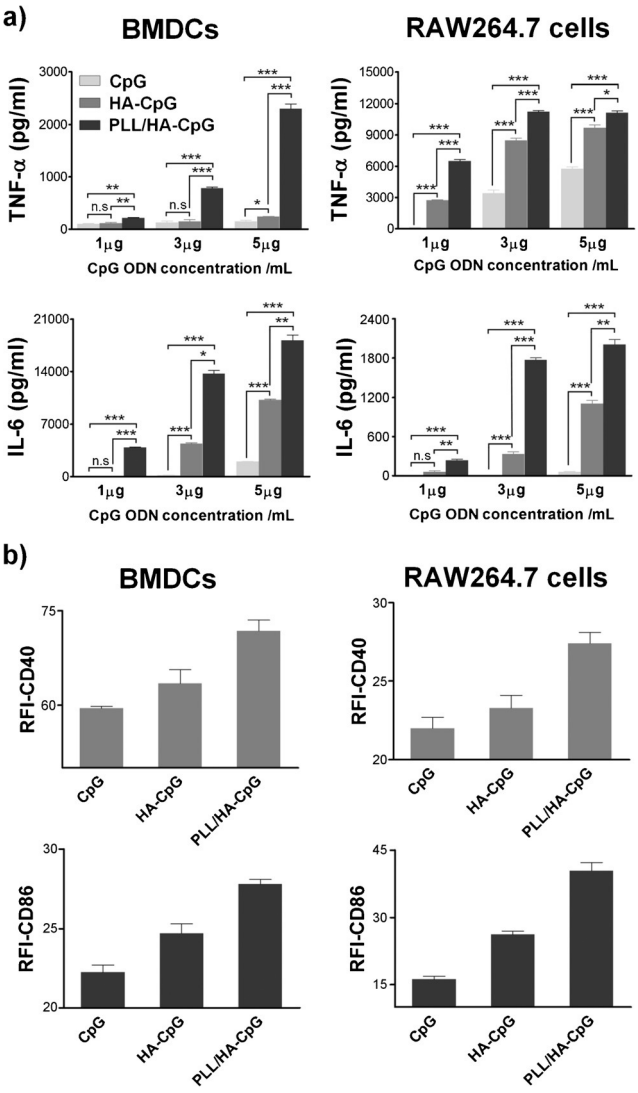

Figure 2. Immunostimulatory effects of multivalent polymer nanocomplexes. a) Cytokine secretion in a concentration-dependent manner by BMDCs and RAW264.7 cells. BMDCs and RAW264.7 cells were incubated with the indicated materials in a CpG concentration-dependent manner for 24 h. The concentration of cytokines in the culture medium was assessed by ELISA. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). b) Expression of the maturation marker. BMDCs and RAW264.7 cells were incubated with the indicated materials at a CpG concentration of 5 μg mL^{-1} for 24 h. The CD40 and CD86 markers were assessed by flow cytometry analysis of the relative fluorescence intensity (RFI).

secretion of cytokines (Figures 2 and S8). When we consider the previous cellular uptake results, no difference in the fluorescence intensity was observed between free CpG and the multivalent HA-CpG nanoconjugates. However, the cytokine expression elicited by the multivalent HA-CpG nanoconjugate was higher than that elicited by free CpG, when same amount of CpG was used. It should be emphasized that the cytokine expression was significantly higher when the BMDCs and RAW264.7 cells were treated with the multivalent PLL/HA-CpG nanocomplex. For example, the secretion of TNF-α was dramatically increased even when low dose (1 μg of CpG) of PLL/HA-CpG nanocomplex was used to treat RAW264.7 cells (74.5 times higher than that of CpG-treated cells). The increase of IL-6 was also dominant when the BMDCs were treated with 3 μg of PLL/HA-CpG nanocomplex (77.3 times higher than that of CpG-treated cells). These results suggest that the enhanced immunostimulatory activity of the multivalent PLL/HA-CpG nanocomplex was synergistically elicited both by the continuous stimulation of endosomal TLR9 through multivalency and by the enhanced cellular uptake of immune-stimulating CpG (Figure S9). Because CpG molecules are conjugated on HA polymer or encapsulated in the PLL/HA nanocomplex, both the direct contact with enzymes such as nucleases can be avoided during the intracellular delivery process. In the acidic endosome, the electrostatic interactions between HA and PLL start to decrease and the multivalent HA-CpG was loosened or separated from the PLL/HA-CpG nanocomplex, resulting in interaction with TLR9 receptor. The secretion of cytokines by BMDCs and macrophages was increased in a concentration-dependent manner in the presence of both the multivalent HA-CpG nanoconjugates and the PLL/HA-CpG nanocomplexes (Figure 2a). These results suggest that an enhanced immunostimulatory effect can be induced even with less CpG (i.e., dose sparing) if we adopt multivalent CpG nanoconjugates and/or nanocomplexes. Therefore, the chemical strategy of synthesizing nanostructured materials by mimicking biological mechanisms that can increase cellular interaction and entry is a promising approach to enhance immunostimulation with minimal side effects. To investigate the maturation effect of multivalent HA-CpG nanoconjugates and PLL/HA-CpG nanocomplexes, we analyzed the expression of maturation markers using flow cytometry (Figure 2b). The expression of the co-stimulatory molecules CD40 and CD86[16] was higher on the immune cells treated with multivalent HA-CpG nanoconjugates or PLL/HA-CpG nanocomplexes than on those treated with free CpG. These results suggest that pathogen-mimicking multivalency and cell entry moieties can enhance not only the activation but also the maturation of immune cells, as an immune-boosting material. To assess the migration of maturing DCs to the lymph node, we employed in vivo tracking of DCs to the lymph nodes using a near-infrared (NIR) optical imaging system. The DCs treated with the indicated materials and indocyanine green (ICG) were injected into the footpads of mice. The NIR signals were detected in the popliteal lymph nodes from 24 h to 48 h (Figure 3a,b). The DCs treated with PLL/HA-CpG nanocomplexes generated the strongest NIR signals, which suggests that DCs activated with the PLL/HA-CpG nanocomplexes were the most efficient at migrating to the draining lymph nodes.

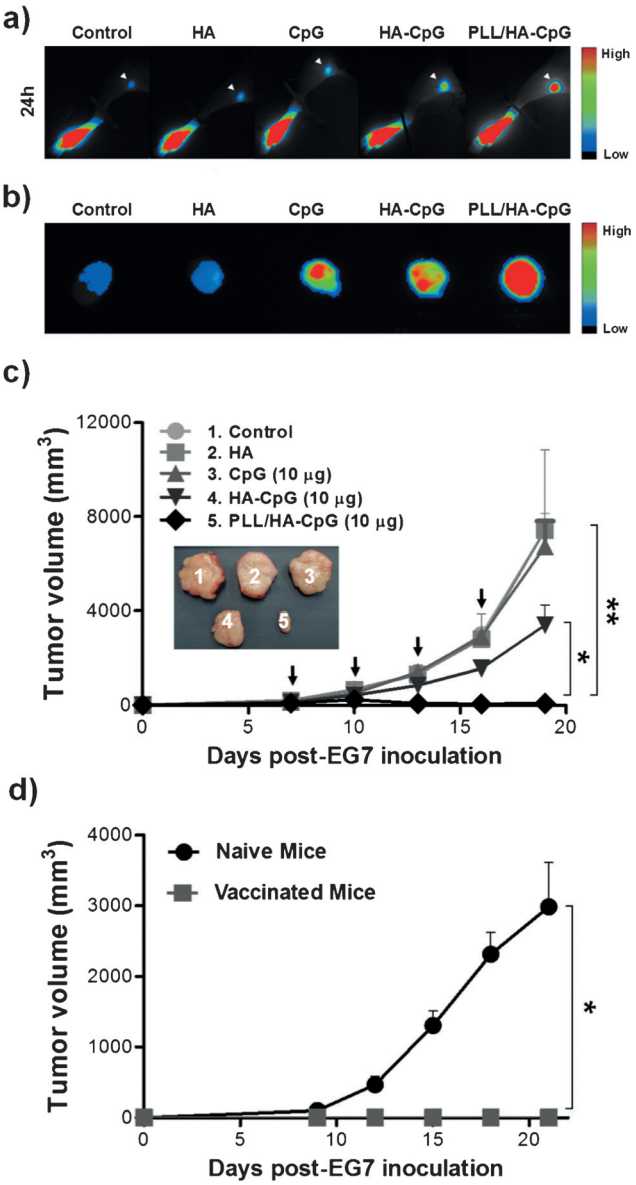

Figure 3. In vivo antitumor and memory response. a) In vivo tracking of DC migration to lymph nodes. NIR imaging of BMDCs treated with the indicated materials at 24 h after injection into the left footpads of mice (triangle: lymph node). b) NIR images of a dissected popliteal lymph node at 48 h. c) Tumor volume until day 19 after injection with the indicated materials; the injection day points are indicated by black arrows. d) Tumor volume until day 21 after tumor rechallenge. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Inspired by the ex vivo immunostimulating capacity of the PLL/HA-CpG nanocomplexes, we investigated the in vivo tumor therapeutic effect. We intratumorally injected the indicated materials into EG7-OVA-tumor-bearing mice four times in two-day intervals. As shown in Figure 3c, CpG alone was insufficient to inhibit tumor growth similar to the control group. However, HA-CpG nanoconjugate or PLL/HA-CpG nanocomplex led to significant inhibition of tumor growth. Notably, the PLL/HA-CpG nanocomplex also led to a drastic

inhibition of tumor growth and full recovery. We have also investigated a systemic immune memory response, which is crucial in tumor therapy to prevent tumor metastasis and relapse.[17] A tumor rechallenge experiment was performed on the PLL/HA-CpG nanocomplex-treated mice that had completely recovered from the primary tumor challenge to confirm the presence of an antitumor memory response (Figure 3d). Analysis of the tumor volume revealed a significant inhibition of secondary tumor growth in the mice vaccinated with the PLL/HA-CpG nanocomplexes in contrast to age-matched naïve mice. Such inhibition in tumor growth explains the generation of systemic tumor-specific immune response in the mice after treatment with PLL/HA-CpG nanocomplex. It should be emphasized that the rational design and chemical synthesis of polymer nanocomplexes bearing multivalent immunostimulatory ligands led to the enhanced stimulation of immune cells in vitro and enhanced antitumor and systemic immune memory response in vivo.

In summary, we have developed HA polymer-based multivalent nanoconjugates and nanocomplex materials for enhanced immunostimulation through the combination of multivalent immune adjuvants with CpG ODNs (as a TLR9 ligand) and cationic PLL (for the enhancement of cellular uptake). These multivalent HA-CpG nanoconjugates efficiently stimulated antigen-presenting cells such as dendritic cells and macrophages. Finally, the multivalent PLL/HA-CpG nanocomplexes resulted in enhanced cellular uptake efficiency and consequently continuous stimulation of endosomal TLR9. The mice vaccinated with dendritic cells treated with the nanocomplexes exhibited tumor growth inhibition as well as a strong antitumor memory response. We demonstrated that multivalent nanoconjugates and nanocomplexes exhibited potent immunostimulatory activity both in vitro and in vivo. The multivalent polymer nanocomplex material revealed in this manuscript can be used to induce antigen-specific immune response after encapsulation of a tumor-specific antigen. In the future, these findings can be used to develop prophylactic or therapeutic cancer vaccines that can recruit and activate immune cells in vivo.[18]

Keywords: cancer therapy · immune cells · immunity · multivalent ligands · nanocomplexes

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