Chemical Communications

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Supporting Information

Rod-shaped and Fluorine-substituted Hydroxyapatite Free of Molecular Immunopotentiator Stimulates Anti-cancer Immunity *in vivo*

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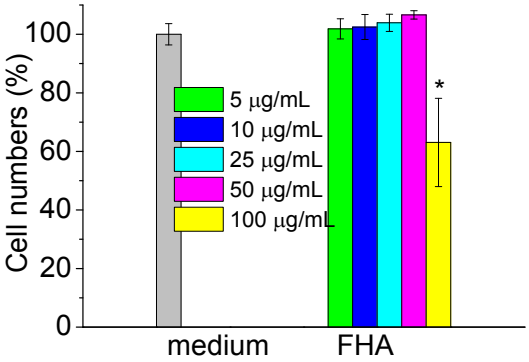

Figure S1. Viability of NIH3T3 cells after exposed to culture medium with different concentration of FHA nanoparticles for 3 days (*, $p<0.05$ compared with medium).

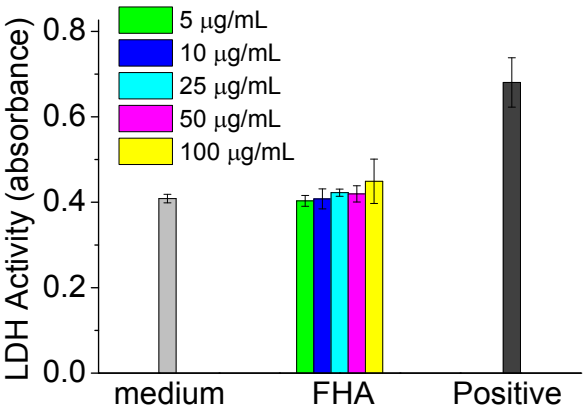

Figure S2. LDH cytotoxicity of NIH3T3 cells after exposed to culture medium with different concentration of FHA nanoparticles for 3 days.

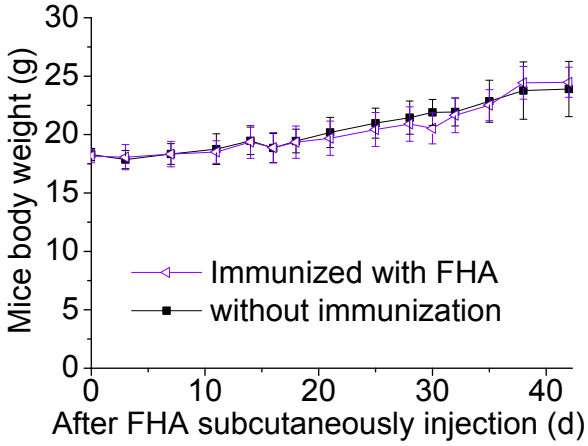

Figure S3. Mice with and without FHA subcutaneous injection showed comparable body weight.

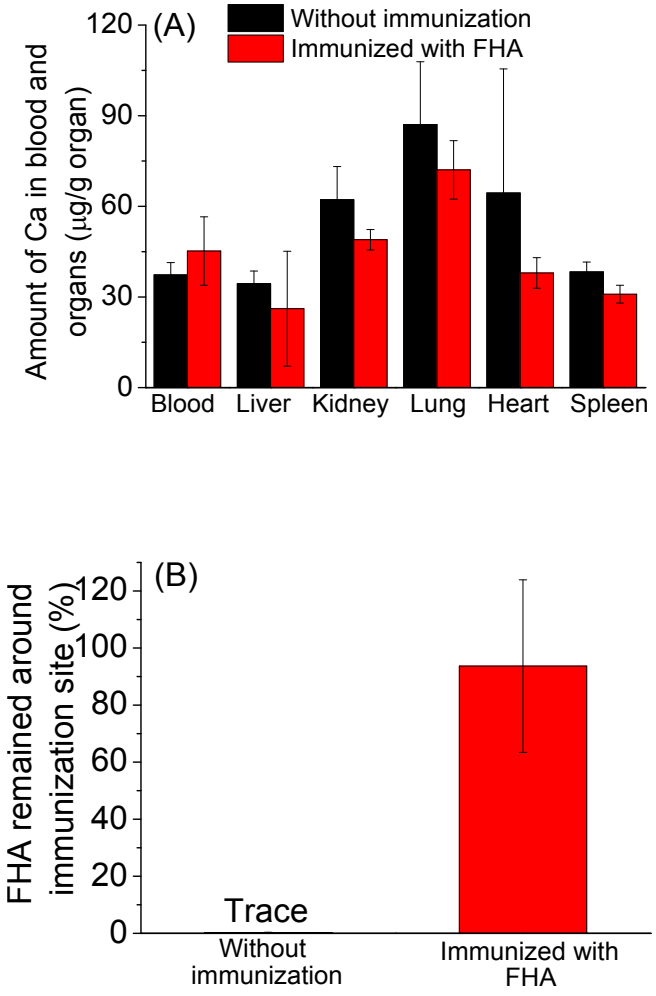

Figure S4. No obvious FHA accumulation in blood, liver, kidney, lung, heart and spleen after 3 times FHA subcutaneous injection (A); most of FHA still remained around injection site after 3 times FHA subcutaneous injection (B).

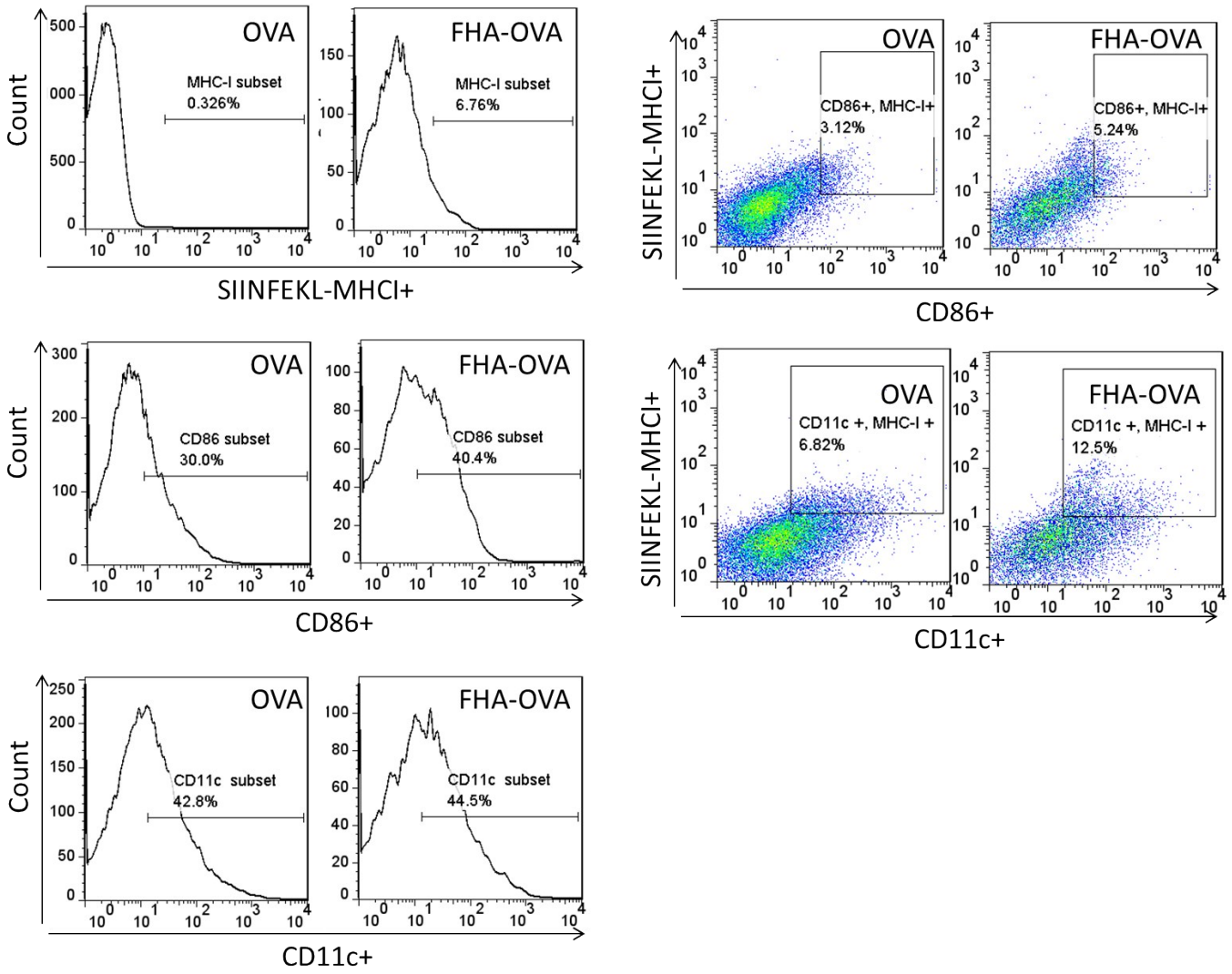

Figure S5. Rod-shaped FHA nanoparticles promote DCs accumulation and cross-presentation of SIINFEKL-MHCI with DCs around injection site *in vivo* (representative results).

Experimental section

Characterization of FHA nanoparticles: The morphologies of FHA nanoparticles were observed using a transmission electron microscope (TEM) and a field emission scanning electron microscope (FE-SEM). The phase composition of FHA nanoparticles was analyzed using a powder X-ray diffractometer. Fourier transform infrared (FTIR) spectrum was recorded using an FTIR-350 spectrometer by the KBr pellet method. Zeta potential was analyzed using a Delta Nano C Particle Analyzer by dispersing particles in calcium- and magnesium-free phosphate buffered saline (PBS(-)).

Fluorescein conjugates of ovalbumin (F-OVA) adsorption: Alum, HA and FHA nanoparticles (25 μg) were mixed with 1 mL of 5 μg/mL F-OVA at 4°C for 12 h. The samples were centrifuged at 10000 rpm for 10 min. The supernatant was used to test F-OVA content by a spectrofluorophotometer (RF-5300, Shimadzu, Japan). The amount of F-OVA adsorbed on alum, HA and FHA nanoparticles was calculated from the decrease of F-OVA concentration before and after adsorption.

***In vitro* test:** *In vitro* cellular uptake, immunogenic activity and cytotoxicity of HA and FHA nanoparticles were evaluated using primary bone marrow dentritic cells (BMDCs). Primary cells are believed to provide more meaningful results than cell lines *in vitro*. BMDCs were harvested using the method described in previous publications [T. Kawashima, et al. Immunity, 2013, 38, 1187-1197]. Typically, bone marrow cells were harvested from femurs of mice. After red blood cell lysis, the residual cells were labeled with phycoerythrin-conjugated anti-CD4, CD8, and I-A/I-E (eBioscience), and then depleted by anti-phycoerythrin magnetic beads (Miltenyi Biotec) and Auto MACS (Miltenyi Biotec). The obtained cells were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum and 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF, Bioreagent). Nonadherent and loosely adherent cells were collected 9 days later. Cell purity of BMDCs was typically 80%–85%, as assessed by staining with fluorescein isothiocyanate-conjugated CD11c (eBioscience) [T. Kawashima, et al. Immunity, 2013, 38, 1187-1197].

For cellular uptake test, the HA and FHA nanoparticles were dispersed in PBS(-) by ultrasonication, and then mixed with F-OVA solution at 4^{o}C for 12h. The mixed samples were added to the medium with a final concentration of 25 μg/mL for particles, and 5 μg/mL for F-OVA, respectively. After 4 h co-culture with BMDCs, the cells were washed twice with PBS(-), stained by 5 μg/mL of Hoechst for cell nuclei and observed by a confocal microscope (Leica TCS SP5, Germany). The fluorescence intensity was quantified by a fluorescent microplate reader (MTP-900, Hitachi, Japan).

For immunogenic activity test, the harvested BMDCs (2×10^5 cells/well) were cultured in RPMI 1640 media with alum, HA and FHA (20-50 μg/mL) particles, respectively. After 24 h of culture, the media were analyzed for IFN-γ and IL-1β concentrations using mouse ELISA kits (BD Pharmingen) according to the manufacturer's instructions. Cell viability of BMDCs was determined using CCK-8 kits (Dojindo Molecular Technologies, Japan).

The cytotoxicity of FHA nanoparticles was evaluated using fibroblastic NIH3T3 cells (NIH3T3-3-4, Riken Bio Resource Center, Japan). 5×10^4 cells·cm^{-2} NIH3T3 cells were precultured for overnight in Dullbecco's modified essential medium supplemented with L-glutamine (0.3 mg/mL) and 10 vol% bovine serum. Then, the culture medium was exchanged with a fresh one containing 5, 10, 25, 50 or 100 ug/mL FHA nanoparticles. The cell culture medium without nanoparticles was used as the negative control. The cell culture medium with 2% Triton X-100 was used as the positive control. After culturing for 3d, the lactate dehydrogenase (LDH) cytotoxicity of the supernatant was tested using a LDH Cytotoxicity Detection Kit (Takara Bio, Inc., Japan); viability of NIH3T3 cells was tested using a CCK-8 kit (Dojindo Molecular Technologies, Japan) in accordance with the manufacturer's instructions.

Antigen-presenting cells accumulation and cross-presentation of anti-SIINFEKL-MHCI antibody: To study the effect of rod-shaped FHA nanoparticles on antigen-presenting cells accumulation and cross-presentation of SIINFEKL-MHCI around injection site *in vivo,* female C57Bl/6J mice were immunized by adjuvant (2 mg/mouse)-OVA (150 μg/mouse) subcutaneously into the left flank at day 0. Then, the mice were immunized by adjuvant (2 mg/mouse) subcutaneously into the left flank of mice at

day 3 and 10. At day 14, the mice were sacrificed, tissue around injection site were harvested to get single cell suspension. The single-cell suspension was washed with PBS containing 0.5% bovine serum albumin (BSA). Non-specific staining was prevented by blocking the cells with anti-CD16/CD32 antibody (2.4G2, BD Biosciences, USA). The cells were stained with the following antibodies for 30 min: anti-mouse CD86, anti-mouse CD11b, anti-mouse CD11c, anti-mouse OVA257-264 (SIINFEKL) peptide bound to H-2Kb. Flow cytometry was performed using FACSAria (BD Bioscience, USA), and data were analyzed using FlowJo software (Tree Star, USA).

Anti-cancer test: Female C57Bl/6J mice were immunized by adjuvant (2 mg/mouse) mixed with OVA (150 μg/mouse) subcutaneously into the left flank at day 0. Then, the mice were immunized by adjuvant (2 mg/mouse) subcutaneously into the left flank of mice at day 3 and 10. At day 14, the mice were challenged by E.G7-OVA subcutaneously into the right flank. Tumor growth on the right flank of mice was monitored for 4 weeks. Mice injected with an established Th1-biased control adjuvant (Poly IC, InvivoGen, 50 μg/mouse) were used as positive controls. In addition, mice injected with FHA (2 mg/mouse) without OVA were used to prove the effect of anti-cancer test was not due to non-specific cytotoxicity of FHA.

To test the distribution of FHA in organs of mice, mice with and without FHA immunization were sacrificed at day 14. The FHA distribution was tested by measuring Ca content by an inductively coupled plasma atomic emission spectrometer (ICP-AES: SPS7800, Seiko Instruments, Inc.)

***Ex vivo* immunogenic activity test:** Female C57Bl/6J mice were subcutaneously immunized with 50 μg of chicken egg ovalbumin (OVA, Sigma-Aldrich) in saline, 2 mg of alum or FHA nanoparticles at the base of the tail and in the two hind footpads. The mice were sacrificed 7 days after immunization. Draining lymph nodes (pooled periaoritic, popliteal, and inguinal lymph nodes) were mechanically minced to make a single cell suspension. Freshly isolated cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were stimulated with or without OVA for 3 days. The amount of cytokines in the supernatant was quantified using mouse IFNγ ELISA kit (BD Pharmingen) according to the manufacturer's instructions.

The animal experiment was permitted by the Ethical Committee of the National Institute of Advanced Industrial Science and Technology (AIST), Japan. All the animal experiments and feeding were carried out in accordance with the guidelines of the Ethical Committee of the National Institute of Advanced Industrial Science and Technology (AIST), Japan.

Statistical analysis: The statistical significance of differences was calculated by One-way ANOVA followed by Tukey Test. A p value of less than 0.05 was considered statistically significant.