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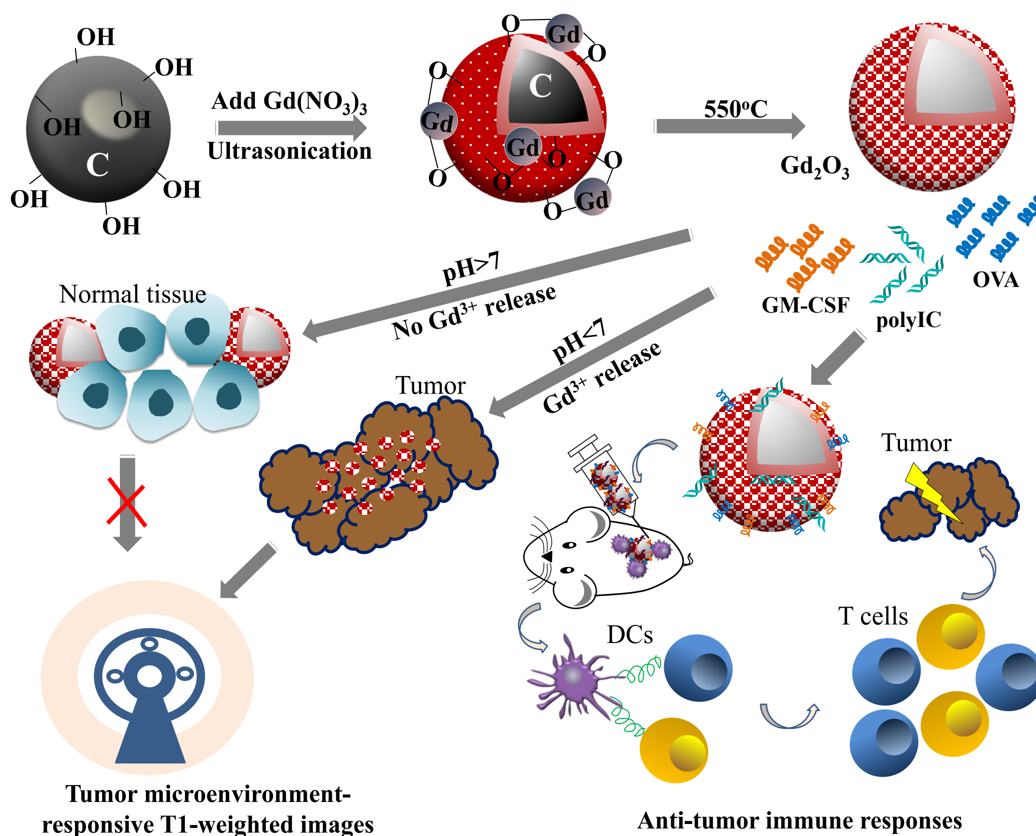

Scheme 1. Schematic illustration of multifunctional hollow Gd_2O_3 nanospheres for cancer immunotherapy and magnetic resonance imaging.

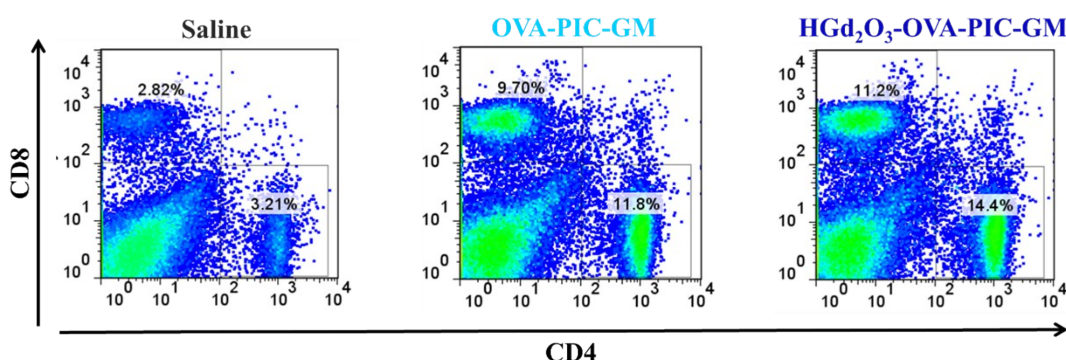

Figure S1. HGd_2O_3-OVA-PIC-GM markedly increases $CD4^+$ and $CD8^+$ T cell population in splenocytes of mice after immunization and challenge with E.G7-OVA cells. Representative results.

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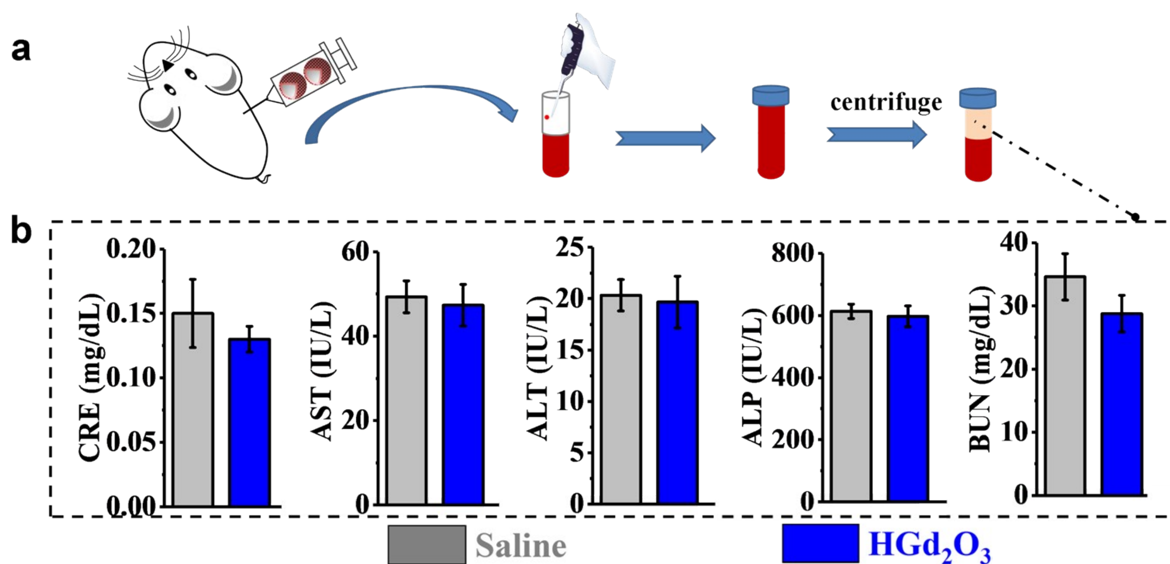

Figure S2. HGd_2O_3 nanospheres show no obvious toxicity as tested by blood biochemistry analysis 7 days after HGd_2O_3 subcutaneously administration.

Experimental Section

Synthesis of template carbonaceous nanospheres

Glucose (Wako) was dissolved in ultrapure water to prepare 0.2 M glucose solution and heated at 180°C for 4 h in a Teflon bottle (80 mL). Carbonaceous nanospheres were collected after centrifugation and rinsed with ultrapure water/ethanol. The obtained product was dried at 80°C.

Synthesis of HGd_2O_3 nanospheres using carbonaceous nanospheres as templates

In a typical method, $Gd(NO_3)_3$ (Wako) was dissolved in ultrapure water to form a 1 M $Gd(NO_3)_3$ solution. Carbonaceous nanospheres (1 g) were dispersed in 50 mL of 1 M $Gd(NO_3)_3$ solution with the assistance of ultrasonication for 4-8 h. The as-prepared product was obtained after centrifugation and rinsed with ultrapure water/ethanol. The obtained product was dried at 80°C and heat-treated at 550°C to prepare HGd_2O_3 nanospheres.

Characterization of HGd_2O_3 nanospheres

The morphology of HGd_2O_3 nanospheres was observed by a field emission scanning electron microscope (FE-SEM, S-4800, Hitachi) and by a transmission electron microscope (TEM, EM-002B, TOPCON). The phase compositions of HGd_2O_3 nanospheres were analyzed by X-ray diffractometry (XRD) employing CuKα X-ray (Model RINT 2500; Rigaku). Particle size distribution was analyzed by a dynamic light scattering photometer (DLS-8000HAL, Otsuka Electronics). Zeta potential was tested by a Delta Nano C Particle Analyzer (Beckman Coulter).

Model antigen adsorption and release from HGd_2O_3 nanospheres

HGd_2O_3 nanospheres (0.5 mg) were mixed with 1mL of F-OVA (0.1 mg/mL, Life Technologies) at 4°C for 24 h. The supernatant was used to test F-OVA content by a fluorescent microplate reader (MTP-

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900, Hitachi). F-OVA loaded onto HGd_2O_3 nanospheres were calculated from the difference of F-OVA concentration before and after adsorption. The F-OVA loaded nanospheres were further dispersed in 1 mL ultrapure water at 37°C with shaking. At predetermined time points, 1 mL of the supernatant from each sample was taken to test F-OVA release. HGd_2O_3 nanospheres loaded with F-OVA were re-dispersed in 1 mL of ultrapure water and shaking by the next predetermined time point.

In vitro **cellular uptake and immunogenic activity test of HGd_2O_3 nanospheres**

In vitro cellular uptake and immunogenic activity tests were performed using primary bone marrow derived DCs of mice. DCs were collected using the method reported in previous paper (*Immunity*, 2013, 38, 1187). For cellular uptake test, HGd_2O_3 nanospheres were dispersed in PBS(-), mixed with F-OVA solution at 4°C for 12 h and added to the medium with a concentration of 25 μg/mL for HGd_2O_3 nanospheres and 5 μg/mL for F-OVA, respectively. After 4-h co-culture with DCs, the cells were washed twice with PBS(-) and tested by a flow cytometry using FACSAria (BD Bioscience) and a fluorescent microplate reader (MTP-900, Hitachi). After overnight co-culture with DCs, the cells were stained by Hoechst (5 μg/mL, Thermo Fisher) for cell nuclei and observed by a confocal microscope (Leica TCS SP5).

For *in vitro* immunogenic activity test, the DCs (2×10^5 cells/well) were cultured in RPMI 1640 media supplemented with HGd_2O_3 nanospheres (50 μg/mL). Three days after culture, the media were analyzed for IL-1β secretion using a mouse ELISA kit (BD Pharmingen).

In vitro **pH-responsive T1-weighted magnetic resonance imaging (MRI)**

HGd_2O_3 nanospheres were dispersed and fixed in PBS(-) containing 3 % agar (pH=7.4) and 10 mM citric acid buffer containing 3 % agar (pH=5.1), respectively. Then, MRI experiments were done on a 2.0-T Biospec 20/30 System with a B-GA20 System (BRUKER, Karlsruhe).

Degradation of HGd_2O_3 in different buffer

The degradation of samples contained in a bag of dialysis membrane in an acetate buffer (pH=5) or a Tris-HCl buffer (pH=7.4) at a particles-to-buffer ratio of 1mg/mL at 37°C with shaker was quantitatively analyzed using an inductively coupled plasma atomic emission spectrometer (ICP-AES: SPS7800, Seiko Instruments). At determined timepoint, all the degradation solutions were collected and same amount of new buffer was added.

In vivo **antigen retention and antigen uptake by DCs around injection site**

All the animal experimental protocols were permitted by the ethical committee on experiments involving animals of the National Institute of Advanced Industrial Science and Technology (AIST), Japan.

In vivo antigen retention around injection site was observed by an IVIS imaging system. Alex Fluor 647-OVA (100 μg/mouse, Molecular Probes)-loaded HGd_2O_3 nanospheres (1 mg/mouse) were subcutaneously injected into the flank of mice (C57BL/6J, female, 6 weeks old, CLEA Inc., Japan) at d0. Alex Fluor 647-OVA remained at injection site was observed by an IVIS imaging system from d0 to d7. The cells around injection site were collected at d7. Non-specific staining was blocked by anti-CD16/CD32 antibody (2.4G2, BD Pharmingen). The cells were stained with anti-mouse CD11c antibody (BioLegend) for 30 min and tested by FACSAria.

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In vivo anti-tumor test and mechanism study

Chicken egg ovalbumin (OVA, Sigma-Aldrich) was used as tumor antigen specific to E.G7-OVA lymphoma cells (CRL-2113™, ATCC®). The flow chart of anti-tumor test was shown in Figure 5a. At first, OVA (100 μg/mouse), polyinosinic-polycytidylic acid (PIC, 50 μg/mouse, InvivoGen) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 1 μg/mouse, InvivoGen) were loaded onto HGd_2O_3 nanospheres (0.3 mg/mouse) and subcutaneously injected into the left flank of mice (C57BL/6J, 6 weeks old, female, CLEA Inc., Japan) at d0, d3 and d10. E.G7-OVA cells (5×10^5 cells/mouse) were subcutaneously injected into the right flank of mice at d14. Tumor size was measured using a caliper.

To study the mechanisms of action of HGd_2O_3 nanospheres to enhance anti-tumor immunity, splenocytes were collected at the endpoint. Non-specific staining was blocked with anti-CD16/CD32 antibody (2.4G2, BD Pharmingen). The cells were stained with anti-mouse CD4 and anti-mouse CD8α antibodies (BioLegend) for 30 min and tested by FACSAria. Inguinal lymph nodes of mice were collected, embedded in paraffin, sectioned and stained by haematoxylin and eosin (HE) at the endpoint. The obtained histological sections were observed by a microscope (BX51, Olympus).

For *in vivo* safety evaluation, HGd_2O_3 nanospheres (0.9 mg/mouse) were injected subcutaneously into the flank of C57/BL6J mice. Blood was collected 7 days later for blood biochemistry analysis.

Statistical analysis

The statistical significance of differences was calculated by log-rank test and student's t-test. A p value of less than 0.05 was considered statistically significant.

Funding Sources

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