Synergistic effects of stellated fibrous mesoporous silica and synthetic dsRNA analogues for cancer immunotherapy

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Table S1 Physical properties of MS and NS nanospheres

<table>
<thead>
<tr>
<th></th>
<th>BET surface area</th>
<th>Average pore diameter</th>
<th>Pore volume</th>
<th>Particle size distribution d10</th>
<th>Particle size distribution d50</th>
<th>Particle size distribution d90</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>252.1 m²/g</td>
<td>-</td>
<td>-</td>
<td>69.9 nm</td>
<td>91.4 nm</td>
<td>142.1 nm</td>
</tr>
<tr>
<td>MS</td>
<td>558.4 m²/g</td>
<td>16 nm</td>
<td>2.3 cm³/g</td>
<td>70.8 nm</td>
<td>97.7 nm</td>
<td>144.0 nm</td>
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</tbody>
</table>

Table S2 Mean time of mice without tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>OVA-PIC</th>
<th>MS-OVA-PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean time of mice without tumor (day)</td>
<td>5.3</td>
<td>20.5</td>
<td>26.9</td>
</tr>
</tbody>
</table>
Figure S1 Representative confocal laser scanning microscope images: F-OVA (a), NS-F-OVA (b), MS-F-OVA (c) after 4 h of culture. (from left to right, bright field, F-OVA, cell nucleus and merged images, respectively)
Figure S2 Stellated fibrous MS significantly promote cellular uptake of a model antigen and BMDC maturation compared with NS and without adjuvant group in vitro. Representative (a) and quantitative (b) results of F-OVA cellular uptake tested by population of F-OVA positive cells; Quantitative results of F-OVA cellular uptake tested by fluorescence intensity (c) after 4 h of culture; Quantitative (d) and representative (e) BMDCs maturation after 1 d of culture (p<0.05).
The stellated MS nanospheres show no obvious toxicity as tested by hematology analysis 7 days after subcutaneous administration (n=3, p>0.05).

Figure S4 Tumor growth curve
Experimental Section

**Synthesis of stellated fibrous mesoporous silica (MS).** The stellated fibrous MS nanospheres were synthesized using a soft-templating method according to previously published protocol with modifications (K. Zhang, et al, *J Am Chem Soc* 2013, **135**, 2427). Typically, hexadecyltrimethylammonium p-toluenesulfonate (CTAT, Sigma-Aldrich) and triethanolamine (TEA, Sigma-Aldrich) were added into ultrapure water with stirring at 70°C. When CTAT and TEA were completely dissolved, tetraethoxysilane (TEOS, Wako) was slowly added to the solution with stirring. The molar ratio of the reaction mixture was 1.00 TEOS: 0.06 CTAT: 0.026 TEA: 80 H₂O, respectively. The reaction mixture was continuously stirred for 2 h to obtain a precipitate. After centrifugation, washing with ultrapure water/ethanol and drying at 80°C, the precipitate was heat-treated at 550°C for 5 h to obtain the stellated fibrous MS nanospheres.

**Synthesis of nonporous silica (NS).** The NS nanospheres were synthesized using the Stöber method with modification (W. Stöber, et al, *J Colloid Interf Sci* 1968, **26**, 62). Typically, H₂O (3 g), CH₃CH₂OH (Wako, 1 g) and NH₃·H₂O (Wako, 28%, 6 g) were mixed and cooled in ice. Then, TEOS (0.3 mL) was added by drop under stir. The mixture solution was stirred for 30 min in ice to obtain a precipitate. After centrifugation, washing with ultrapure water/ethanol and drying at 80°C, the NS nanospheres were obtained.

**Characterization of MS and NS nanospheres.** The morphology of the stellated fibrous MS and NS nanospheres was observed using a field emission scanning electron microscope (FE-SEM, Hitachi). The stellated fibrous MS nanospheres were observed using transmission electron microscope (TEM, JEOL and TOPCON). Particle size distribution of the nanospheres was tested by a dynamic light scattering photometer (Otsuka Electronics). The nanospheres were analyzed by X-ray diffractometry (XRD) employing CuKα X-ray using a powder X-ray diffractometer (Rigaku). Fourier transform infrared (FTIR) spectra of the nanospheres were recorded using an FTIR-350 spectrometer (JASCO) by the KBr pellet method. Zeta potential of the nanospheres was analyzed using a Delta Nano C Particle Analyzer (Beckman Coulter, Inc.)
by dispersing particles in saline and calcium and magnesium free phosphate buffered saline (PBS(-)), respectively. The nitrogen gas ($N_2$) adsorption-desorption isotherm of MS nanospheres was measured by a specific surface area/pore size distribution analyzer (BELSORP, ASAP).

**Biomolecules adsorption and release.** The stellated fibrous MS nanospheres (0.1 mg) and ferritin (model antigen, 2 μL of 100 mg/mL) were added to 1 mL of PBS(-) at room temperature with shaking for 6 h. The product was centrifuged, washed with ultrapure water, and dried at room temperature.

MS and NS nanospheres (0.4 mg) were individually mixed with 2 mL of 0.1 mg/mL Poly(I:C) (InvivoGen) in saline at 4°C for 24 h. Then, the products were centrifuged and the supernatant was used to test Poly(I:C) content by a UV–visible spectrophotometer (Jasco). The amounts of Poly(I:C) adsorbed on the MS and NS nanospheres were calculated from the difference in Poly(I:C) concentrations before and after adsorption.

MS and NS nanospheres (0.5 mg) were individually mixed with 1 mL of 0.1 mg/mL F-OVA (Life Technologies) at 4°C for 24 h. The product was centrifuged and the supernatant was used to test F-OVA content by a fluorescent microplate reader (Hitachi). The amounts of F-OVA adsorbed on the MS and NS nanospheres were calculated from the differences in F-OVA concentration before and after adsorption. Then, the nanospheres adsorbed with F-OVA were 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 the F-OVA release. The MS or NS nanoparticles 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 BMDCs maturation test.** *In vitro* cellular uptake and primary bone marrow derived dendritic cells (BMDCs) maturation tests were evaluated using BMDCs harvested using the method described in previous publication (T. Kawashima, et al, *Immunity* 2013, 38, 1187).
For cellular uptake test, the MS and NS nanospheres were mixed with F-OVA solution at 4°C for 12 h. 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, some wells were washed with PBS(-) and analyzed by an flow cytometry (FACSARia, BD Bioscience). Other wells were quantified by a fluorescent microplate reader (Hitachi). After co-culture with BMDCs overnight, the cells were stained with 5 µg/mL of Hoechst (Thermo Fisher) for cell nuclei and observed by a confocal microscope (Leica).

For BMDCs maturation test, the BMDCs (2×10^5 cells/well) were cultured in RPMI 1640 media with MS and NS nanospheres (20 µg/mL). After 1d of culture, the BMDCs was washed with PBS(-) containing 0.5% bovine serum albumin (Gibco). Non-specific staining was blocked by anti-CD16/CD32 antibody (2.4G2, BD Pharmingen). The cells were stained with anti-mouse CD86 and anti-mouse CD11c antibodies (Biolegend) for 30 min. Flow cytometry was performed using FACSARia.

**In vivo anti-cancer test.** The animal experimental protocol was permitted by the ethical committee on experiments involving animals in the National Institute of Advanced Industrial Science and Technology (AIST), Japan. All the animal experiments and feeding were carried out in accordance with the AIST Guideline for animal experiments.

Chicken egg ovalbumin (OVA, Sigma-Aldrich) was used as the cancer antigen specific to E.G7-OVA lymphoma cells (CRL-2113™, ATCC®). The flow chart of in vivo anti-cancer study was shown in Figure 3 a. At first, the MS-OVA-PIC, prepared by mixing OVA (5 mg/kg), Poly(I:C) (PIC, 0.625 mg/kg, InvivoGen) and MS nanospheres (15 mg/kg) in saline, was subcutaneously injected into the left flank of mice (C57BL/6J, female, 6 weeks old, CLEA Inc., Japan) at d0, d3 and d10 to establish immune response against OVA. For cancer immunotherapy, mice were generally immunized three times to build up immune response against cancer (D. O. Villarreal, et al, Cancer Research, 2014, 74, 1789; Y. Tian, et al, Oncotarget, 2017, 8, 45951; M. Kawahara et al, Int J Oncol, 2013, 43, 2023; C. J. Repique, et al, Infect Immun, 2002, 70, 3318). Mice injected with OVA or OVA-PIC (OVA=5 mg/kg, PIC=0.625 or 2.5 mg/kg) were used as
controls. E.G7-OVA cells (5×10^5 cells/mouse) were subcutaneously injected into the right flank of mouse at d14. Cancer size was measured using a digital Vernier caliper while mice were conscious. Cancer volume was calculated according to 1/2 \times \text{length} \times \text{width}^2.

Flow cytometry analysis was used to study the mechanisms of anti-cancer immunity. Splenocytes were collected from mice 1 month after the immunization. Non-specific staining was blocked by anti-CD16/CD32 antibody (2.4G2, BD Pharmingen). Then, the cells were stained with anti-mouse CD4 and anti-mouse CD8α antibodies (Biolegend) for 30 min. Flow cytometry was performed using FACSaria.

To examine the \textit{in vivo} safety, the MS nanospheres (45 mg/kg) were subcutaneously injected into the left flank of C57/BL6J mice. Blood was harvested for blood hematology analysis 7 days later.