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Amorphous silica nanoparticles enhance cross-presentation in murine dendritic cells

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ABSTRACT

Nanomaterials (NMs) exhibit unique physicochemical properties and innovative functions, and they are increasingly being used in a wide variety of fields. Ensuring the safety of NMs is now an urgent task. Recently, we reported that amorphous silica nanoparticles (nSPs), one of the most widely used NMs, enhance antigen-specific cellular immune responses and may therefore aggravate immune diseases. Thus, to ensure the design of safer nSPs, investigations into the effect of nSPs on antigen presentation in dendritic cells, which are central orchestrators of the adaptive immune response, are now needed. Here, we show that nSPs with diameters of 70 and 100 nm enhanced exogenous antigen entry into the cytosol from endosomes and induced cross-presentation, whereas submicron-sized silica particles (>100 nm) did not. Furthermore, we show that surface modification of nSPs suppressed cross-presentation. Although further studies are required to investigate whether surface-modified nSPs suppress immune-modulating effects *in vivo*, the current results indicate that appropriate regulation of the characteristics of nSPs, such as size and surface properties, will be critical for the design of safer nSPs.

1. Introduction

Nanomaterials (NMs) are substances that have at least one external dimension ⩽100 nm. Due to their small size and large surface-to-volume ratio, NMs show properties that are not found in bulk samples of the same material (particle size >100 nm) such as tissue permeability and biologic reactivity [1,2]. Thus, over the last decade, the use of NMs has increased in products such as cosmetics, foods, and medicines as a way of adding value to new or existing products [3–5].

However, there is now worldwide concern over whether the novel properties of NMs make them unsafe for humans. For example, it has been reported that titanium dioxide nanoparticles ($nTiO_2$) induce DNA damage and genetic instability *in vivo* [6]. Furthermore, we have shown that $nTiO_2$ and amorphous silica nanoparticles (nSPs) induce reproductive toxicity and consumptive coagulopathy in mice [7,8]. Despite intensive research efforts, the relationships between biological responses to and the physicochemical properties of NMs, such as their size and surface properties, are not yet well understood. To fully utilize the potential benefits of NMs, it is crucial that we obtain more information for the design of safer NMs.

Previously, we showed that nSPs—one of the most frequently used NMs in cosmetics and foods [9]—with a diameter of 70 nm (nSP70) penetrate the skin barrier and enter dendritic cells (DCs) in the skin in mice [10,11]. Because DCs are central orchestrators of immunity that provide necessary innate signals and adaptive functions through the processing and presentation of antigens to which T cells respond [12,13], it is possible that nSPs have immune-modulating effects and are associated with the development of immune diseases. We further showed that co-administration of nSPs plus protein-antigen enhances antigen-specific $CD8^+$ T cell response and aggravates immune diseases in mice [14,15]. $CD8^+$ T cell response is only activated if

Abbreviations: DCs, dendritic cells; IL-2, interleukin 2; NMs, nanomaterials; nSPs, amorphous silica nanoparticles; $nTiO_2$, titanium dioxide nanoparticles; OVA, ovalbumin; SPs, amorphous silica particles; SR, scavenger receptor.

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antigen-presenting cells, mainly DCs, present antigens on their major histocompatibility complex (MHC) class I molecules [16]. However, most exogenous antigens, which are introduced into DCs by the endocytic pathway, are not presented on MHC class I molecules [17]; most exogenous antigens are presented on MHC class II molecules [18]. Because nSPs enhance the $CD8^+$ T cell response against exogenous antigen, nSPs might affect antigen processing and presentation in DCs. We hypothesize that nSPs promote the presentation of exogenous antigen on MHC class I molecules, through a pathway termed cross-presentation, which subsequently induces an immune-modulating effect.

Here, we investigate the influence of nSPs on antigen presentation via cross-presentation in DCs.

2. Materials and methods

2.1. Silica particles

Unmodified amorphous silica particles (SPs) with diameters of 70, 100, 300, or 1000 nm (designated nSP70, nSP100, nSP300, and mSP1000, respectively) and nSP70 modified with surface carboxyl groups (nSP70-C) or amine groups (nSP70-N) were purchased from Micromod Partikeltechnologie (Rostock/Warnemünde, Germany). Silica particle suspensions were stored at room temperature. The suspensions were sonicated and then vortexed for 1 min immediately prior to use. The physicochemical properties of these SPs have been described previously [10,15,19].

2.2. Regents

Polyinosinic acid (Poly I), chloroquine, and lactacystin were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Cell culture

DC2.4 cells, which were previously characterized as immature DC cells from C57BL/6 (H-2 Kb) [20], were kindly provided by Dr. Kenneth L. Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA, USA) and maintained in RPMI-1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS, 10 ml/L 100× nonessential amino acid solution (Gibco, Invitrogen, Carlsbad, CA, USA), 50 μM 2-mercaptoethanol (Gibco), and 1% antibiotic cocktail (Nacalai Tesque, Kyoto, Japan). CD8-OVA1.3 cells, a T–T hybridoma against the $OVA_{257–264}$/MHC class I molecule (H-2 Kb) complex [21], were kindly provided by Dr. Clifford V. Harding (Case Western Reserve University, Cleveland, OH, USA) and maintained in Dulbecco's modified Eagle's medium (D-MEM; Wako) supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics cocktail. Cell viability was determined by using a WST-8 assay kit (Nacalai Tesque).

2.4. Cross-presentation assay

DC2.4 cells were seeded at a density of 5×10^4 cells/well in a 96-well flat-bottom culture plate (Nunc, Roskilde, Denmark) and incubated overnight at 37 °C (95% room air, 5% CO_2). Each well was then washed with PBS and the cells pulsed with 2.9–15 μg/mL of one of the silica particle suspensions and 100 μg/mL chicken egg ovalbumin (OVA; Sigma–Aldrich), or 100 μg/mL OVA alone. After 24 h, each well was washed with PBS and the cells fixed with 0.05% glutaraldehyde (Wako). The cells were then co-cultured with 1×10^5 cells/well of CD8-OVA1.3 cells for 24 h. The amount of interleukin 2 (IL-2) released into an aliquot of culture medium (200 μL) was measured by using a murine IL-2 ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

To study the antigen presentation pathway, DC2.4 cells (5×10^4 cells/well) were incubated with 100 μg/mL Poly I, 50 μM chloroquine, or 2.5 μM lactacystin for 1 h prior to the addition of OVA and one of the nSPs. In these assays, because the inhibitors are cytotoxic, we pulsed the cells with nSP70 (40 μg/mL) and OVA (100 μg/mL) in the continued presence of inhibitor for only 6 h. After incubation, the cells were washed and fixed, and 1×10^5 cells/well of CD8-OVA1.3 cells was added. After 24 h, the IL-2 levels in the supernatants were measured as described above.

2.5. Statistical analysis

All data are presented as mean ± SD. Differences were compared by using Student's *t*-test or Dunnett's test after ANOVA. Differences between the experimental groups and the control group were considered significant at $P < 0.05$.

3. Results and discussion

3.1. nSPs enhance cross-presentation

Here, we used nSPs with diameters of 70 and 100 nm (nSP70 and nSP100, respectively) and conventional SPs with diameters of 300 and 1000 nm (nSP300 and mSP1000, respectively). We previously confirmed that the hydrodynamic diameters of these particles, as measured by means of a dynamic light scattering system, were 76, 106, 264, and 1136 nm, respectively [15]. The size distribution spectrum of each silica particle showed a single peak and the hydrodynamic diameter corresponded almost exactly to the primary particle size for each sample, indicating that the silica particles used in this study are well-dispersed in solution [10,15]. In addition, transmission electron microscopy (TEM) images confirmed that the particles are well-dispersed smooth-surfaced spheres, as described previously [10].

To examine whether nSPs affect antigen processing and presentation in DCs and enhance cross-presentation, we first assessed the correlation between particle size and cross-presentation. DC2.4 cells were incubated with the SPs and OVA, and then co-cultured with CD8-OVA1.3 cells. When CD8-OVA1.3 cells recognize $OVA_{257–264}$/H-2 Kb complexes and become stimulated, they begin to produce IL-2. The amount of IL-2 in the supernatant correlates with the frequency of OVA presentation on MHC class I molecules; therefore, we assessed the cross-presentation of OVA by determining the amount of IL-2 released into an aliquot of culture medium. IL-2 production in the nSP70- and nSP100-treated groups was increased in a dose-dependent manner and was significantly higher than that in the OVA alone group (Fig. 1). In contrast, IL-2 production in the nSP300- and mSP1000-treated groups was the same as that in the OVA alone group (Fig. 1). These results suggest that nSPs influence antigen processing and induce cross-presentation in DCs.

3.2. nSP70 induces cross-presentation via the cytosolic pathway and is dependent on nSP70 uptake via scavenger receptors

There are two main intracellular pathways of cross-presentation: the cytosolic pathway and the vacuolar pathway [16,22,23]. In cross-presentation via the cytosolic pathway, internalized antigens enter the cytosol via endosomes, which are then degraded by proteasomes. Proteasome-generated peptides then feed into the classical MHC class I-mediated antigen presentation pathway in the same way as endogenous antigens. In contrast, in the vacuolar pathway, antigens are degraded within

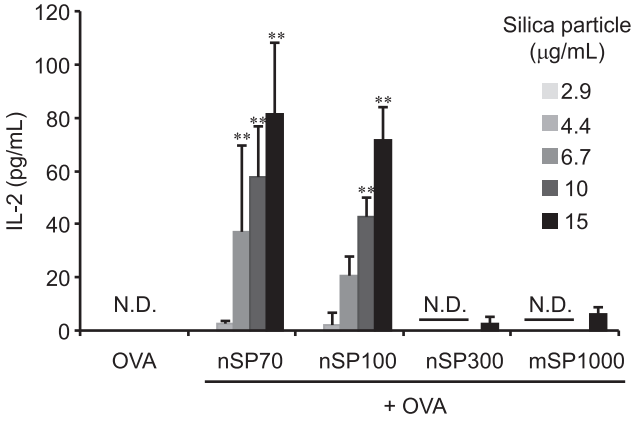

Fig. 1. The effect of different-sized silica particles on OVA presentation on MHC class I molecules in DC2.4 cells. DC2.4 cells were pulsed with OVA alone or OVA plus one of the sizes of silica. After silica exposure, the DC2.4 cells were co-cultured with CD8-OVA1.3 cells. The concentration of IL-2 in the supernatants was measured. The data are presented as the mean ± SD for three independent cultures ($n = 3$). N.D. not detected. $^{**}P < 0.01$ versus OVA alone group by Dunnett's test.

the endosomes through endosomal acidification, and both antigen processing and loading onto MHC class I molecules occur within endocytic compartments. To determine which pathway is involved in nSP70-induced cross-presentation, we examined the effect of the potent proteasome inhibitor lactacystin or the endosomal acidification inhibitor chloroquine on nSP70-induced cross-presentation. Chloroquine treatment did not inhibit nSP70-induced IL-2 production (Fig. 2A), whereas lactacystin treatment strongly inhibited nSP70-induced IL-2 production (Fig. 2B). These results suggest that nSP70 induces cross-presentation via the cytosolic pathway.

Next, we analyzed the effect of cellular uptake of nSP70 on the induction of cross-presentation. Because some reports have shown that scavenger receptors (SRs) are related to the uptake of crystalline silica particles and nSPs [24,25], we examined nSP70-induced cross-presentation after treatment with Poly I, which is an SR inhibitor. nSP70-induced cross-presentation was completely inhibited by treatment with Poly I (Fig. 2C); therefore, nSP70-induced cross-presentation is dependent on SRs.

3.3. Effect of nSP70 surface modification on cross-presentation

Recent articles have focused on the possible influence of surface charge on the *in vivo* biodistribution, cellular uptake, and/or cytotoxicity of nanoparticles [7,26,27]. Taken together, these studies have shown that the surface properties of nSPs could be important for the development of safer nSPs. To investigate the effect of surface modification of nSP70 on cross-presentation, we evaluated cross-presentation in DC2.4 cells treated with OVA and nSP70 modified with either surface amine groups (nSP70-N) or carboxyl groups (nSP70-C). As mentioned above, we confirmed through TEM that nSP70-N and nSP70-C are smooth-surfaced, spherical particles, and that surface modification changes the surface charge of the particles [7]. IL-2 production in the OVA and nSP70-N- or nSP70-C-treated groups was not enhanced compared with the OVA only group; however, it was enhanced in the OVA and nSP70-treated group (Fig. 3). These results indicate that nSPs modified with surface amine groups or carboxyl groups do not induce

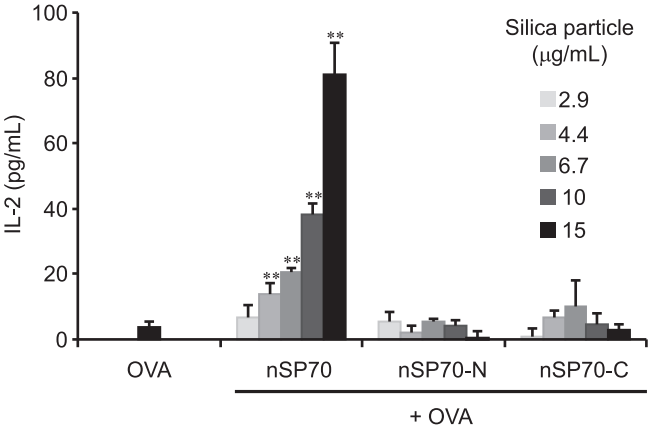

Fig. 3. Relationship between the surface physicochemical properties of nSP70 and nSP70-induced cross-presentation. DC2.4 cells were pulsed with OVA alone or OVA plus one of the nSP70s. After silica exposure, the DC2.4 cells were co-cultured with CD8-OVA1.3 cells for 24 h. The concentration of IL-2 in the supernatants was measured. The data are presented as the mean ± SD for three independent cultures ($n = 3$). $^{**}P < 0.01$ versus OVA alone group by Dunnett's test.

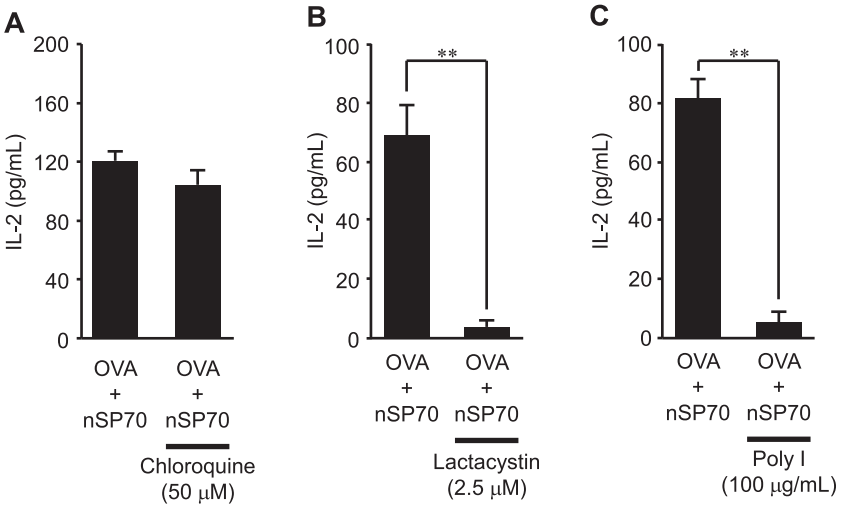

Fig. 2. Effects of various inhibitors on nSP70-induced cross-presentation. DC2.4 cells were pretreated with or without 50 μM chloroquine (A), 2.5 μM lactacystin (B), or 100 μg/mL Poly I (C). After 1-h incubation, DC2.4 cells were pulsed with OVA alone or OVA plus nSP70. After nSP70 exposure, DC2.4 cells were co-cultured with CD8-OVA1.3 cells. The concentration of IL-2 in the supernatants was measured. The data are presented as the mean ± SD for three independent cultures ($n = 3$). $^{**}P < 0.01$ versus OVA alone group by Student's *t*-test.

cross-presentation. Therefore, appropriate regulation of the surface properties of nSPs may produce safer nSPs that do not induce cross-presentation and immune-modulation.

It is important to clarify why nSP70 induces cross-presentation yet nSP70-N and nSP70-C do not. It has been reported that the production of reactive oxygen species (ROS) induces the rupturing of endosomal/lysosomal membranes [28,29]. We previously reported that nSPs with a diameter $\leqslant$ 100 nm strongly induce endocytosis-dependent ROS generation [30]. It is therefore possible that nSP70 induces ROS generation dependent on SRs, which causes phagosomal destabilization and subsequently more antigen enters the cytosol. Furthermore, we observed that in the murine macrophage cell line Raw264.7, nSP70-N and nSP70-C induce little ROS production compared with nSP70 (data not shown). Although further analysis of the relationship between ROS production and nSP-induced cross-presentation is needed, it is possible that only unmodified nSPs induce ROS production and enhance cross-presentation.

In the current study, we showed that nSPs with diameters between 70 and 100 nm enhance cross-presentation. Furthermore, we showed that surface modification of nSPs with amine or carboxyl groups results in little cross-presentation. Although further studies are required to investigate whether surface modification of nSPs suppresses immune-modulating effects *in vivo*, these results indicate that appropriate regulation of nSP size and surface properties may be crucial for the design of safer nSPs. Furthermore, accurate regulation of the ability of nSPs to induce cross-presentation may lead to new applications for nSPs, such as in cancer vaccines. We believe a detailed analysis of the mechanisms of nSP-mediated cross-presentation will be invaluable for both the design of safe nSPs and the development of new applications for them.

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