Impact of Surface Modification on Cellular Uptake and Cytotoxicity of Silica Nanoparticles

Sandra Vranic  
Tokyo University of Science

Eri Watanabe  
Tokyo University of Science

Kayoko Miyakawa  
Tokyo University of Science

Sakie Takeuchi  
Tokyo University of Science

Yurika Osada  
Tokyo University of Science

Sahoko Ichihara  
Jichi Medical University

Cai Zong  
Tokyo University of Science

Wenting Wu  
Nagoya University

Toshihiro Sakurai  
Tokyo University of Science

Akira Sato  
Tokyo University of Science

Yasushi Hara  
Tokyo University of Science

Toshihiro Suzuki  
Tokyo University of Science

Ryo Abe  
Tokyo University of Science

Sonja Boland  
University of Paris

Lang Tran  
Institute of Occupational Medicine

Gaku Ichihara (✉ gak@rs.tus.ac.jp)  
Tokyo University of Science  https://orcid.org/0000-0001-5707-5300
Abstract

**Background:** Silica nanoparticles (SiO$_2$ NPs) are widely used in industrial products as additives for rubber and plastics or as filler strengthening concrete, as well as being used in the biomedical field for drug delivery and theranostic purposes. The present study investigated the effects of amino or carboxyl functionalization of rhodamine-labeled SiO$_2$ NPs on cellular uptake and cytotoxicity.

**Methods:** Male mice were randomly divided into seven groups (n=6, each) and exposed to non-functionalized (plain), carboxyl or amino-functionalized rhodamine-labeled SiO$_2$ NPs at 2 or 10 mg/kg bw, or endotoxin-free water, by pharyngeal aspiration. At 24 hours after administration, the mice were euthanized and bronchoalveolar lavage fluid (BALF) was collected for differential cell count and identification of silica nanoparticle uptake using confocal microscopy. In the *in vitro* studies, murine RAW264.7 macrophages were exposed to non-functionalized, amino- or carboxyl-functionalized Rhodamine-labeled SiO$_2$ NPs. Nonspecific caspase inhibitor and necrostatin-1 were used to determine the involvement of caspase or receptor-interacting protein 1 kinase domain in the cytotoxicity.

**Results:** The *in vivo* study demonstrated that the neutrophil and macrophage counts and the percentage of macrophages with internalized particles was highest in the order of carboxyl >= amino- > non-functionalized particles. The *in vitro* study demonstrated greater cytotoxicity for non-functionalized silica nanoparticles, compared to the others. Treatment with non-specific caspase or necroptosis inhibitor did not attenuate MTS cytotoxicity of non-functionalized silica nanoparticles.

**Conclusion:** We conclude that carboxyl-functionalized SiO$_2$ NPs are internalized by macrophages more efficiently but less cytotoxic than plain SiO$_2$ NPs. The cytotoxic effect of plain SiO$_2$ NPs, which cannot be explained by apoptosis or necroptosis, can be avoided by carboxyl- or amino- functionalization.

Introduction

Nanoparticles (NPs) can be potentially used in various applications in the fields of nanotechnology, research and medicine. The small particle size and unique chemical and physical properties are highly exploitable in different consumer products as well as in the biomedical field, such as drug delivery vectors, imaging purposes and/or cell tracking [1–4]. However, their low biodegradability, potential inhalation or prolonged skin contact with nanoparticles based on the small diameter and high surface reactivity are considered drawbacks for wider usage [4]. Therefore, before the application of NPs becomes widely accepted, it is necessary to establish their toxicological profile and understand the potential adverse effects that might arise from human and environmental, accidental or desired exposure.

Silicon dioxide (silica) nanoparticles (SiO$_2$ NPs) are nowadays widely used in industrial products as additives for rubber and plastics or as strengthening fillers for concrete. In addition, they are used in the biomedical field for drug delivery or theranostic purposes [5–9]. The toxicological profile of SiO$_2$ NPs has been widely studied recently, with increasing body of literature on the potential adverse effects of SiO$_2$ NP
exposure. Silica materials exist in both crystalline and amorphous forms. The most common form of crystalline silica is quartz, whose toxicity has been studied for many years and is linked with chronic bronchitis, emphysema and silicosis [9–11]. Compared to the crystalline SiO$_2$ NPs, the amorphous form is less toxic [5, 6]. With regard to the mechanism of toxicity, in vitro studies have shown that the toxic effect of SiO$_2$ NPs is mainly mediated by induction of oxidative stress and activation of intrinsic or mitochondrial (caspase-activated) apoptotic pathways [12–17]. Reactive oxygen species (ROS)-mediated cell death is considered one of the main mechanisms of action of many different types of nanomaterials (NMs), including silica NPs. In contrast, the results of a recent study indicate that total silanol content, cell membrane damage, and cell viability mediate the toxicity of SiO$_2$ NPs, rather than intracellular ROS [18]. The majority of the in vivo toxicological data are based on acute exposure studies, which usually include intra-tracheal instillation, intravenous injection or oral exposure [5, 6]. Submicron amorphous silica particles were found to have greater inflammatory and cytotoxic potential compared to their bigger counterparts [19]. In the study of Morris et al. [20], C57BL/6 mice were intratracheally instilled with 4 or 20 mg SiO$_2$ NPs/kg body weight and significant effects were observed only at the high dose of 20 mg/kg. Twenty hours after instillation, approximately 20- and 10-fold increase in the cell number was observed in the bronchoalveolar lavage (BAL) of mice treated with the bare and amine-coated SiO$_2$ NPs, respectively, compared to the control mice; neutrophils were also increased about 30- and 20-fold, respectively [20]. Other studies demonstrated acute and chronic exposure to SiO$_2$ NPs aggravated airway inflammation [21–25].

One of the strategies to build “safe by design” NPs is to apply various types of surface modifications to coat the NPs and modulate their surface reactivity, thereby decreasing their toxic effects. For example, surface modification of SiO$_2$ NPs was found to reduce their aggregation and nonspecific binding [26], while functionalization with amino or phosphate groups was shown to mitigate their pro-inflammatory and immunomodulatory effects in allergic airway inflammation [21]. Interestingly, coating of SiO$_2$ NPs with polyethylene glycol polymer (PEG) was not found to be efficient in reducing the pro-inflammatory potential of these NPs in vivo [21, 27]. Furthermore, few in vitro studies have shown that surface modification of SiO$_2$ NPs reduces their potential for inflammasome activation and cytotoxicity [28, 29].

With this in mind, the present study was designed to determine the effect of surface modification of 25 nm amorphous SiO$_2$ NPs both in vivo (C57BL/6JJcl mice) and in vitro (murine macrophage RAW 264.7 cell line), with a special emphasis on their pro-inflammatory and cytotoxic potentials. Our data support the hypothesis that surface modification of SiO$_2$ NPs can modulate the uptake properties of the material, their pro-inflammatory potential and cytotoxicity. We showed that exposure to bare SiO$_2$ NPs with terminal Si-OH group decreased the number of macrophages collected from bronchoalveolar lavage fluid (BALF) in vivo. On the other hand, both carboxyl- and amino-functionalized SiO$_2$ NPs were efficiently taken up by macrophages or neutrophils in vivo, accompanied by increase in the number of neutrophils collected from BALF at the high dose used (10 mg NPs/kg bw). The results showed that SiO$_2$ NPs cytotoxicity is not related with internalization of SiO$_2$ NPs and neutrophil infiltration, although
internalization of SiO2 NPs might be linked to neutrophil infiltration. The results of in vitro and in vivo experiments were complementary to each other, underlining the importance of surface modification of SiO2 NPs in modulating their toxicological profile, and suggesting that functionalization of 25 nm SiO2 NPs with carboxyl groups in consumer products or for biomedical applications could lead to efficient cellular uptake, and that functionalization with the amino or carboxyl group reduced the toxicity of SiO2 NPs.

Materials And Methods

Silica nanoparticles

Rhodamine-labeled SiO2 NPs, “Sicastar”, of 30 nm in diameter, functionalized with carboxy (Cat. #40-02-301), amino group (Cat. #40-01-301) or non-functionalized (Cat. #40-00-301) were purchased from Micromod Partikeltechnologie, Rostock, Germany. All NPs were dispersed in water at 25 mg/mL. The size of the NPs in water or in complete cell culture medium was characterized by DLS, Zeta potential analysis and TEM. Zeta-potential was measured with Photal LEZA-600 (Otsuka Denshi Co., Osaka). The fluorescence intensities of the three types of silica NPs were measured at different concentrations using ARVO™MX 1420 Multilabel Counter (Perkin Elmer, Waltham, MA). The slopes of the regression lines for independent variable of concentration and dependent variable of fluorescence intensity were calculated to obtain the relative fluorescence intensity between different types of silica NPs labeled with rhodamine (Supplementary file 1).

Animals

Forty-two male C57BL/6JJcl mice of 7-week-old were purchased from CLEA Japan Inc (Tokyo, Japan). All mice were housed and acclimatized to the new environment for one week in a pathogen-free animal room controlled at 23–25 °C and 55–60% humidity. Light was set within a 12-h light-dark cycle (on at 09:00 and off at 21:00), and food and water were provided ad libitum. This study was conducted according to the Japanese law on the protection and control of animals and the Animal Experimental Guidelines of Tokyo University of Science. The experimental protocol was also approved by the Animal Ethics Committee of Tokyo University of Science prior to the experiment.

Mice were randomly divided into seven groups of six each and exposed to plain SiO2 NPs, carboxyl SiO2 NPs or amino SiO2 NPs at 2 or 10 mg/kg bw, which were equivalent to 40 or 200 µg per mouse if body weight was 20 g, or endotoxin-free water. These exposure levels were half of those adopted in a previous study, which demonstrated that exposure to SiO2 NPs by intratracheal instillation increased the number of macrophages in BALF at 0.5 mg silica/mouse (20 mg/kg bw) but not at 0.1 mg silica/mouse (4 mg/kg bw) (Morris 2016). With regard to the relation of the above exposure levels by intratracheal instillation to those by inhalation, one study showed that 690 µg of titanium dioxide deposited in the rat lung after exposure to ultrafine titanium dioxide at 125 mg/m³ for 2 hours (Osier 1997). Since the alveolar surface areas of rat and mice are 2970 [30] and 82.2 cm² [31], respectively, the estimated lung deposition of
titanium dioxide in mice was $690 \times \frac{82.2}{2970} = 19 \mu g$ when exposed to ultrafine titanium dioxide at $125 \text{ mg/m}^3$ for 2 hours. The higher dose of 200 µg in the present study is comparable to ultrafine particles deposited in the lungs after inhalation exposure to ultrafine particles at $125 \text{ mg/m}^3$ for 21 hours. SiO$_2$ NPs dispersed in water at 25 mg/mL were vortexed and then further diluted with endotoxin-free water to obtain the NP solution at 1 and 5 mg/mL.

Mice were anesthetized with pentobarbital and then exposed to 40-µl aliquot of samples of SiO$_2$ NPs by pharyngeal aspiration, as described previously (Gabazza et al. 2004, Wu et al. 2015). The technique of pharyngeal aspiration involved placement of the nanoparticle suspension on the back of the tongue followed by pulling of the tongue to induce a reflex gasp with resultant aspiration of the droplets. At 24 h after administration, the mice were euthanized by intraperitoneal injection of pentobarbital. BAL was performed by cannulation of the trachea with 18-gauge needle, and infusion and collection of 5/6 mL of saline was repeated six times.

**Total and differential cell counts in BALF**

The recovered BALF was centrifuged (1,500 rpm, 5 min, 4 °C), and the cell pellet was mixed with 1 mL of ACK lysing buffer (Gibco-Thermo Fischer Scientific, Waltham, MA) for hemolysis. Ten mL of Dulbecco’s phosphate buffer saline (DPBS) was added and centrifuged at 1,500 rpm and 4 °C. The resultant pellet was re-suspended in DPBS for total and differential cell counts. The total cell count was measured using hematocell counter. Aliquots of $5 \times 10^4$ cells in 400 µl DPBS per slide were prepared for cytospins. The cell mixture was added to EZ Single Cytofunnel®(Thermo, UK and centrifuged for 10 min at 1,000 rpm with Cytospin, using cytoslides. The slides were dried overnight at room temperature and then stained with the Differential Quik Stain Kit (Modified Giemsa, Sysmex Co., Kobe, Japan) for differential cell count in 10 fields (20 x magnification) of each slide.

**Fluorescence immunocytology**

A slide obtained by Cytospin was washed in DPBS three times and incubated with blocking agent (1% BSA) for one hour. The slide was further incubated with Biotin anti-Ly6G and Ly6C (Gr-1) (BD, Franklin Lakes, NJ), which was diluted 400 folds in 1% BSA, for one hour at room temperature, washed in DPBS three times and then incubated with 200-fold diluted Cy5 streptavidin (BioLegend, San Diego, CA) for 30 min at room temperature to stain the neutrophils. Ly6G and Ly6C (Gr-1) were used as markers of neutrophils, as their expression levels are known to correlate with differentiation and maturation of granulocytes [32, 33] and are only expressed transiently on bone marrow cells in the monocyte lineage [33]. The slides were counterstained with Hochest33342 for 10 min at room temperature and enclosed with Fluorescent Mounting Media (Dako, Agilent, Santa Clara, CA). Cells and SiO$_2$ NPs were observed with confocal microscope Fv10i (Olympus, Tokyo).

**Cell culture**

Murine macrophages RAW 264.7 cell were kindly provided by Prof. Kenneth Dawson, University of College Dublin and grown in Dulbecco’s Modified Eagle Medium (DMEM, high glucose) with L-glutamine and
phenol red (Wako, Cat. #044-29765) supplemented with 1 mM sodium pyruvate (100 mM, Gibco, Cat. #11360-070), 100 U/mL penicillin, 100 mg/mL streptomycin and 250 ng/mL amphotericin B (anti-anti, Gibco, Cat #15240-062), 2 mM glutamine and 10% FBS. All experiments were performed with cells from passages 4 to 15. Cells were grown in T25-flasks (Violamo, AS ONE Co., Osaka) in monolayers. Exponentially growing cells were maintained in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C and were passaged once every two days using a cell scraper (NEST 100071).

**NP treatment**

Depending on the experiment and after reaching 70–80% confluence, the cells were seeded onto appropriate cell culture plates and treated with silica nanoparticles of different surface modification. Before exposure to NP, the cells were rinsed with PBS to eliminate trace amounts of FBS. Treatments were performed under FBS-free condition for two reasons: 1) serum is reported to modulate NP uptake [34], and 2) to mimic in vivo condition whereby bronchial cells are not directly exposed to serum proteins. Stock of 30 nm rhodamine-labeled silica nanoparticles (25 mg/mL in water) was vortexed shortly before the preparation of the final dilution for the treatment.

**MTS assay**

MTS assay was conducted using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) as described previously [35] and the instructions provided by the manufacturer. Briefly, RAW264.7 cells were seeded at $1.5 \times 10^4$ cells/well onto 96-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO$_2$ and 95% air for 24 hours. After incubation, the cell culture medium was removed from each well with a multichannel pipette, and the cells were washed three times with DPBS to remove FBS. The cells were incubated for 4 or 24 hours with the three types of silica nanoparticles dispersed in FBS-free cell culture medium at a final concentration ranging from 0.3 to 30 µg/cm$^2$. After incubation with the silica nanoparticles, the cells were washed twice with 1xDPBS, and incubated with MTS reagent (1.4 mL CellTiter 96® AQueous One Solution Reagent and 7.1 mL of complete phenol red-free cell culture medium with FBS). Cellular viability was determined by measuring absorbance at 490 nm, which reflected the reduction of {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (MTS) to formazan by mitochondria in viable cells.

**Confocal microscopy**

Cells were seeded in 8 well Lab-TekII™ chambered cover glasses (Nunc, Thermo Scientific, Dominique Dutscher, Brumath, France) at 16,590 cells/well in complete cell culture medium, and then incubated for 24 h. Upon 70–80% confluence, the cells were washed three times with DPBS to remove FBS and treated with 0.3 mL/well of NPs at pre-selected concentrations for 24 h at 37 °C and 5% CO$_2$. After removal of medium, the cells were washed one time and fixed in 4% PFA for 20 min at 25 °C, rinsed twice with DPBS and then incubated with Cell Mask Green Plasma Membrane Stain (C37608 Thermo Fisher Science, Waltham, MA) and Hoechst for 10 min. The cells were washed with DPBS twice. After embedding the cells in Mounting Medium, they were observed by confocal microscopy (model FV.10, Olympus, Tokyo). The maximum concentration of silica nanoparticles $35.2 \times 10^5$ µg/cell (8.40 µg/cm$^2$) was determined to
be equivalent to exposure level of 40 µg/mouse in vivo, given that the average number of macrophages collected in BALF was 1.14 × 10^5 cells/mouse.

**Flow cytometry**

RAW264.7 cells were seeded onto 6-well plates at 2.37 × 10^4 cells/cm^2 in complete cell culture medium and incubated for 24 h before treatment. After treatment with 3.0 mL/well of silica NPs at the preselected concentrations for 1 or 4 h in dark, the medium was removed, cultures were thoroughly washed with PBS three times and treated with 0.1% trypan blue for 1 min to quench the fluorescence of rhodamine on the cell surface. The concentration of trypan blue for quenching the fluorescence of rhodamine was determined beforehand by plot of trypan blue and rhodamine intensity in RAW264.7 cells exposed to bare rhodamine-labeled SiO_2_ NPs (Supplementary Fig. 2). The cells were washed with PBS, mixed with 500 µL of FACS buffer (PBS containing 0.5% FBS and 0.1% NaN_3) and harvested by cell scraper. Cell-associated fluorescence was detected using FACSCompur™ and results were analyzed with FlowJo software (BD, Franklin Lakes, N.J). The Mean Fluorescence Intensity (MFI) of rhodamine (excitation 488 nm, Filter range 564–606 nm) from three different size fractions indicated by the forward scatter (FS) was shown by the flow cytometer. The results are reported as the median of the distribution of cell fluorescence intensity obtained by analyzing cells in the gate. To adjust the differences in the fluorescence intensity relative to weight, the intensity of rhodamine fluorescence was measured at different concentrations of three types of SiO_2_ NPs using ARVOMx-fla system (485 nm/535 nm 1.0 s).

**LDH cytotoxicity assay**

LDH cytotoxicity assay was conducted using Pierce LDH cytotoxicity assay kit following the instructions provided by the manufacturer (Thermo Fisher Scientific). Briefly, RAW264.1 cells were plated at 10^4 cells/well in 100 µl of medium in a 96-well tissue culture plate. To minimize the cytotoxic effect of lack of FBS, DMEM was replaced by Opti-MEM for LDH cytotoxicity assay and further investigation of the effect of pan-caspase or necroptosis inhibitor. After incubation at 37 ºC under 5% CO_2_ for 24 hours, the cells were exposed to bare, carboxyl-functionalized, amino-functionalized rhodamine-labeled SiO_2_ NPs at 5.85 µg/cm^2 (19.5 µg/mL). After the exposure for 1, 4, 12, 24, 36 and 48 hours, the supernatant of the culture medium was collected by centrifugation, and dispensed at 50 µl/well into another 96-well plate. Each cell was incubated in the presence of 50 µl of LDH reaction mixture at room temperature for 30 min in dark. The reaction was stopped by adding 50 µl of stop solution, and absorbance at 490 and 680 nm was read by Plate reader Gen5 (BioBik, Osaka, Japan).

**Evaluation of effects of caspase inhibitor Z-VAD-FMK**

Z-VAD-FMK, {Benzyloxy carbonyl-L-valyl-L-alanyl-[((2S)-2-amino-3-(methoxycarbonyl)propionyl)]fluoromethane, cell-permeable pan-caspase inhibitor was dissolved at 20 mM in DMSO and diluted to 20 or 40 µM by culture medium Opti-MEM® | Reduced Serum Media (Thermo Fisher Scientific). RAW264.1 cells were preincubated with Z-VAD-FMK at 20 or 40 µM or vehicle for one hour, and exposed to plain rhodamine-labeled silica nanoparticles at 28 µg/mL for 18 hours in
Opti-MEM®. Cytotoxicity was evaluated using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) as mentioned above.

**Evaluation of effects of necroptosis inhibitor necrostatin-1**

Necrostatin-1 or necrostatin-1 inactive control (Cayman Chemical, Ann Arbor, MI) was dissolved in 20 mM in DMSO and diluted to 10 or 20 µM by culture medium Opti-MEM® | Reduced Serum Media (Thermo Fisher Scientific). Raw264.1 cells were preincubated with Z-VAD-FMK at 10 or 20 µM or vehicle for one hour, and exposed to plain rhodamine-labeled silica nanoparticles at 8.4 µg/cm² (28 µg/mL) for 18 hours in Opti-MEM®. Cytotoxicity was evaluated using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) as mentioned above.

**Quantitative real time PCR**

RAW264.1 cells were seeded onto 6-well plates at 23,700 cells/cm² and exposed to silica NPs at the concentration of 0.33, 1.67 and 8.37 µg/cm² (1.06, 5.30, and 26.50 µg/mL, respectively). The cells were collected by centrifugation at 1,000 rpm for 5 min at 4°C. Total RNA from the cells was isolated by using ReliaPrepTM RNA Cell Miniprep System (Promega, Fitchburg, WI). The concentration of total RNA was quantified by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, DE). RNA was reverse transcribed to single-strand cDNA using SuperScript III First-Strand Synthesis System for RT-PCXR (Life Technologies). The cDNA was subjected to quantitative PCR analysis with Thunderbird cyber green master mix (TOYOBO, Osaka, Japan) and primers designed by TAKARA (Kusatsu, Japan). The primers are 5'-GTCCCTCAACGGAAGAACCAA-3' (forward) and 5'-TCTCAGACAGCGAGGCACAT-3' (reverse) for MIP-2, 5'-GATCGGTCCCCAAAGGGATG-3' (forward) and 5'-GTGGTTTGTGAGTGTGAGGGT-3' (reverse) for TNFα and 5'-GATCATTGCTCCTCCTGAGC-3' (forward) and 5'-ACTCCTGCTTGTGATCCCA-3' (reverse) for β-actin.

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Differences with the control or between groups were analyzed respectively by Dunnett's or Tukey's multiple comparison following one-way ANOVA. A probability (p) of < 0.05 denoted the presence of a statistically significant difference. For the analysis of relative fluorescence intensity among different types of particles, regression lines were obtained by forcing the intercept to zero using Excel 2016 (Microsoft, Redmond, WA). All statistical analyses were performed using JMP (version 14, SAS Institute, Cary, NC).

**Results**

**Physicochemical properties of SiO₂ NPs**

We studied first the in vitro and in vivo effects of the three types of 25 nm SiO₂ NPs. For this purpose, we used plain SiO₂ NPs with hydroxyl group (OH-SiO₂ NPs), and carboxyl- or amino-modified SiO₂ NPs.
(COOH- SiO₂ NPs or NH₂- SiO₂ NPs). The diameter and surface charge of these NPs were determined by DLS and Zeta potential measurements both in water (since the materials were dispersed in endotoxin-free, ultra-pure water for intra-tracheal instillation in mice lungs) and in the cell culture medium relevant to the exposure of RAW264.8 cells. As indicated in Table 1, the mean diameters of the carboxyl and amino modified, and OH-SiO₂ NPs in water were 33.9 ± 0.1 nm, 34.8 ± 0.2 nm and 34.1 ± 0.2 nm, respectively, indicating a mono-dispersed size distribution of NPs. Similarly, the mean diameters of the three types of SiO₂ NPs in the cell culture medium were comparable, with 33.6 ± 0.6, 33.6 ± 0.3 and 36.3 ± 0.3 nm for the carboxyl and amino modified, and plain NPs, respectively. Interestingly, regardless the surface modification of these NPs using three different functional groups, their overall surface charge was neither modified in water nor in the cell culture medium (Table 1). As indicated, the mean surface charges of COOH-, NH₂- and OH-SiO₂ NPs in water were −25.6 ± 4.05, -41.5 ± 3.02 and −33.2 ± 1.85 mV, respectively, while the respective values in the cell culture medium were −23.5 ± 4.0 mV, -23.8 ± 2.5 mV and −23.3 ± 1.9, respectively. That the mean surface charge was more negative than −20 mV indicates good colloidal stability of SiO₂ NPs both in water and in the cell culture medium.

### Table 1
Physicochemical properties of SiO₂ NPs dispersed in water and in complete cell culture medium, studied by DLS and Zeta potential measurement.

| Type of SiO₂ NPs | Solvent               | Diameter (nm) | Zeta potential (mV) |
|------------------|-----------------------|---------------|---------------------|
| NH₂-SiO₂ NPs     | Water                 | 33.9 ± 0.1    | -25.6 ± 4.05        |
| COOH-SiO₂ NPs    | Water                 | 34.8 ± 0.2    | -41.5 ± 3.02        |
| OH-SiO₂ NPs      | Water                 | 34.1 ± 0.2    | -33.2 ± 1.85        |
| NH₂-SiO₂ NPs     | FBS-free medium       | 33.6 ± 0.6    | -23.5 ± 3.96        |
| COOH-SiO₂ NPs    | FBS-free medium       | 33.6 ± 0.3    | -23.8 ± 2.54        |
| OH-SiO₂ NPs      | FBS-free medium       | 36.3 ± 0.3    | -23.3 ± 1.87        |

Data are mean ± SD of 4 and 3 measurements of diameter and zeta potentials, respectively.

**In vivo toxicity**

In order to address the *in vivo* toxicity of the three types of SiO₂ NPs, we measured body and lung weights of the test animals as a rough indication of disturbance of body and lung homeostasis following instillation of NPs. We also collected BALF for total and differential cell counts, which are reliable markers of lung inflammation induced by NPs. In order to better understand the localization of NPs after the
instillation procedure, the macrophages and neutrophils were differentially stained and examined by confocal microscopy.

**Body and lung weight**

There was no significant difference in body weight between vehicle-treated control mice and mice treated with any of the three types of SiO$_2$ NPs at 2 or 10 mg NPs/kg bw (Table 2). On the other hand, there was a significant increase in the lung weight in the OH-SiO$_2$NP -treated mice at 2 and 10 mg/kg bw.

| Table 2 | Weight of body and lungs of mice at 24 h after exposure to SiO$_2$NP by pharyngeal aspiration. |
|---------------------------------|-------------------------------------|-----------------|-----------------|
|                                | Concentration of SiO$_2$ NPs (mg/kg bw) | n  | Body weight (g) | Lung weight (mg) |
| Vehicle                        | 0                                   | 6  | 20.9 ± 1.4      | 293 ± 22 |
| NH$_2$-SiO$_2$ NPs             | 2                                   | 6  | 21.6 ± 0.4      | 301 ± 5  |
|                                | 10                                  | 6  | 21.3 ± 1.4      | 321 ± 23 |
| COOH-SiO$_2$ NPs               | 2                                   | 6  | 21.6 ± 1.2      | 305 ± 16 |
|                                | 10                                  | 6  | 21.5 ± 1.0      | 322 ± 6  |
| OH-SiO$_2$ NPs                 | 2                                   | 6  | 21 ± 1          | 324 ± 25*|
|                                | 10                                  | 6  | 20 ± 1          | 331 ± 16*|

*P < 0.05, compared with the vehicle control, by Dunnett’s multiple comparison following ANOVA.
Table 3
MIP-2 mRNA level relative to β-actin in RAW264.1 cells exposed to different SiO2NPs.

| n | Concentration of SiO₂ NPs (µg/cm²) | 0      | 0.33   | 1.67   | 8.34   |
|---|------------------------------------|--------|--------|--------|--------|
|   |                                     |        |        |        |        |
| 3 h exposure                          |        |        |        |        |        |
| Vehicle                               | 3      | 0.33 ± 0.11 |
| NH₂- SiO₂ NPs                         | 3      | 0.54 ± 0.17 | 1.02 ± 0.09 | 2.40 ± 0.11 |
| COOH- SiO₂ NPs                        | 3      | 0.61 ± 0.20 | 0.89 ± 0.16 | 1.61 ± 0.15 |
| OH-SiO₂ NPs                           | 3      | 0.40 ± 0.07 | 1.82 ± 0.48 | 166 ± 30* |
| 6 h exposure                          |        |        |        |        |        |
| Vehicle                               | 3      | 0.04 ± 0.01 |
| NH₂- SiO₂ NPs                         | 3      | 0.05 ± 0.01 | 0.16 ± 0.09 | 0.55 ± 0.11 |
| COOH- SiO₂ NPs                        | 3      | 0.08 ± 0.02 | 0.18 ± 0.01 | 0.53 ± 0.12 |
| OH-SiO₂ NPs                           | 3      | 0.09 ± 0.06 | 1.65 ± 0.29 | 25 ± 29* |

*P < 0.05, compared with the vehicle control, by Dunnett’s multiple comparison following ANOVA.
Table 4
TNF-α mRNA level relative to β-actin in RAW264.1 cells exposed to different SiO₂ NPs.

| n | Concentration of SiO₂ NPs (µg/cm²) | 0 | 0.33 | 1.67 | 8.34 |
|---|----------------------------------|---|------|------|------|
| 3 h exposure | | | | | |
| Vehicle | 3 | | | | |
| NH₂-SiO₂ NPs | 3 | | | | |
| COOH-SiO₂ NPs | 3 | | | | |
| OH-SiO₂ NPs | 3 | | | | |
| 6 h exposure | | | | | |
| Vehicle | 3 | | | | |
| NH₂-SiO₂ NPs | 3 | | | | |
| COOH-SiO₂ NPs | 3 | | | | |
| OH-SiO₂ NPs | 3 | | | | |

*P < 0.05, compared with the vehicle control, by Dunnett’s multiple comparison following ANOVA.

**BALF total and differential cell count**

Treatment with different types of SiO₂ NPs at 2 mg/kg bw did not increase the total cell number (Fig. 1A). On the other hand, treatment with amino- and carboxyl-functionalized SiO₂ NPs at 10 mg/kg bw induced increase in BALF total cell count. Interestingly, there was no change in the total cell number after treatment with hydroxyl functionalized SiO₂ NPs at 10 mg/kg bw. Further analysis showed treatment with 10 mg/kg bw OH-SiO₂ NPs decreased the total number of BALF macrophages, compared to the untreated group (Fig. 1B). On the other hand, 10 mg/kg bw carboxyl functionalized SiO₂ NPs increased the number of macrophages (Fig. 1B), and carboxyl- or amino-functionalized SiO₂ NPs increased BALF neutrophils (Fig. 1C). The increase in the numbers of total cells and neutrophils was higher with COOH-SiO₂ NPs than NH₂-SiO₂ NPs (Fig. 1A and C). Hydroxyl (plain) SiO₂ NPs increased the number of neutrophils at 2 mg/kg bw, but had no such effect at 10 mg/kg bw (Fig. 1C).

**Interaction of SiO₂ NPs with BALF macrophages and neutrophils**
COOH- and NH$_2$-SiO$_2$ NPs were both similarly internalized by cells regardless their dose, while only a few OH-SiO$_2$ NPs made contact with the cells (Fig. 2A). Internalization of NH$_2$- and COOH-SiO$_2$ NPs into the macrophages was not influenced by their dose (persistently ~ 60%), while only around 40% of the macrophages internalized OH-SiO$_2$ NPs (Fig. 2B and D). On the other hand, internalization of carboxyl and amino functionalized SiO$_2$ NPs into the neutrophils was dose-dependent (30–40% of cells internalized SiO$_2$ NPs at 10 mg/kg bw, Fig. 2C and E), with the numbers and percentages of such neutrophils significantly higher at 10 mg/kg bw of each of the above two types, compared to those neutrophils with internalized hydroxyl functionalized SiO$_2$ NPs (only 10% at 10 mg/kg bw).

**In vitro toxicity of NPs**

**MTS cell viability assay of SiO$_2$ NPs in RAW264.7 macrophage cell line**

The MTS assay was used to assess the cytotoxic potential of SiO$_2$ NPs, in which tetrazolium salts are reduced to formazan by metabolically active cells (probably through NAD(P)H-dependent dehydrogenases), producing measurable color changes proportionate to the number of viable cells. The percentage of live cells is then estimated by measuring absorbance of formazan at 490 nm, wavelength that is clearly distinguishable from rhodamine absorption profile. Treatment of the cells with OH-SiO$_2$ NPs, but not NH$_2$- and COOH-SiO$_2$ NPs, significantly decreased cell viability in time- and dose-dependent manners (Fig. 3). Exposure to OH-SiO$_2$ NPs significantly decreased cell viability at ≥ 3 µg/cm$^2$ (≥ 10 µg/ml) after 4 h of treatment and at ≥ 7.5 µg/cm$^2$ (≥ 25 µg/ml) after 24 h of treatment. The decrease in cell viability was also dose-dependent, with < 40% of the cells remaining metabolically active after 4 h exposure to 30 µg/cm$^2$ (100 µg/ml) of OH-SiO$_2$ NPs (Fig. 3A). The decrease in cell viability was also time-dependent; <40% of the cells was still alive and metabolically active after 24 h of treatment with 7.5 µg/cm$^2$ (25 µg/ml) OH-SiO$_2$ NPs (Fig. 3B). The above *in vitro* data support the hypothesis that OH-SiO$_2$ NPs is the most toxic among the three types and suggest that the observed decrease in BALF macrophages (Fig. 1B) was mediated through cell death. Similarly, this cytotoxic effect of OH-SiO$_2$ NPs could explain the observed increase in BALF neutrophil count, compared to the control, though it but reached a plateau at ≥ 2 mg/kg bw (Fig. 1C), resulting in lack of statistical different in BALF total number of cells in the untreated mice. These data highlight the need to perform BALF differential cell count in order not to overlook subtle effects of these NPs on different cells in the lung.

**Interaction of SiO$_2$ NPs with RAW264.8 macrophage cell line**

Next, we assessed the interaction of the three types of NPs with RAW264.8 macrophages in order to understand the mechanism of OH-SiO$_2$ NPs toxicity. COOH- SiO$_2$ NPs were efficiently internalized by
RAW264.8 macrophages from 1 h after exposure, in dose- and time-dependent manners (based on the higher amounts of internalized NPs in RAW264.8 macrophages after 4 and 24 h of treatment, Fig. 4A). Interestingly, hydroxyl plain NPs mainly co-localized with the plasma membrane irrespective of the dose used (Fig. 4A). This was remarkable already after 1 h of treatment at the highest concentration of NPs and even more prominent after 4 and 24 h of treatment.

Internalization of NPs by cells was further quantified by flow cytometry (Fig. 4B and Suppl Fig. 1). To eliminate the signal coming from NPs adsorbed on the surface of the cells, 0.1% trypan blue was added shortly before the analysis. The working concentration of trypan blue was optimized prior to the experiment (Suppl Fig. 1). Flow cytometry showed three populations of RAW264.4 cells (Suppl Fig. 2). Due to difference in the slopes of the regression lines, with rhodamine intensity as the dependent variable and SiO₂ NPs concentration as the independent variable (Suppl Fig. 3), the fluorescence of COOH-SiO₂ NPs and OH-SiO₂ NPs was each divided by the ratio of the slope for COOH-SiO₂ NPs and OH-SiO₂ NPs to the slope for NH₂-SiO₂ NPs for normalization. Exposure to COOH-SiO₂ NPs increased the mean value of the normalized fluorescence intensity in the population gated to A (Fig. 4B), B and C (Suppl Fig. 4) in dose- and time-dependent manners. Exposure to NH₂- or OH-SiO₂ NPs increased the mean of normalized intensity in all examined populations to an extent lesser than exposure to COOH-SiO₂ NPs.

**Cytotoxicity of OH-SiO₂ NPs**

The LDH cytotoxicity test showed higher LDH release from the cells exposed to OH-NPs compared to the other types of NPs (Suppl Fig. 5). Pretreatment with pan caspase or necroptosis inhibitor did not attenuate the decrease in MTS cell viability induced by exposure to OH-SiO₂ NPs for 18 h (Fig. 5A and B), suggesting that the cytotoxicity of OH-SiO₂ NPs is unrelated to apoptosis or necroptosis.

**SiO₂ NPs-induced RAW264.8 macrophage-related pro-inflammatory response**

Finally, we assessed the pro-inflammatory potential of the three types of NPs by measuring the expression of two genes by RT-qPCR. At 8.34 µg/cm², OH-SiO₂ NPs increased the expression of both MIP-2 genes at 3 and 6 h post-exposure (Table 2). The same NPs also increased the expression of TNF-α at 3 h post-treatment at 8.34 µg/cm² and at 6 h post-treatment at 1.67 µg/cm². Exposure to at 8.34 µg/cm² COOH-SiO₂ NPs also increased the expression of TNF-α at 6 h post-treatment.

**Discussion**

The results of the *in vivo* study showed that COOH-SiO₂ NPs increased BALF neutrophils to the greatest extent among the three types of SiO₂ NPs. This increase was accompanied by a higher rate of COOH-SiO₂ NPs internalized into BALF neutrophils compared with NH₂- or OH-SiO₂ NPs. On the other hand, OH-SiO₂ NPs reduced the number of BALF macrophages in a dose-dependent manner. In the in vitro arm of the study, we confirmed the uptake of COOH-SiO₂ NPs by RAW 264.7 macrophage cells by flow cytometry,
and that the extent of such uptake was greater than that of NH$_2$- or plain SiO$_2$ NPs. Toxicity analysis using the MTS assay and LDH cytotoxicity test showed the highest toxicity of plain SiO$_2$ NPs among three types of SiO$_2$ NPs. The cell toxic effect was not accompanied by recovery of cell viability by pan-caspase inhibitor or necroptosis inhibitor, suggesting that the cell death was necrotic in nature. Taking the results of both the in vivo and in vitro studies together, we can conclude that exposure to OH-SiO$_2$ NPs induces cell death of macrophages, while exposure to the carboxyl- or amino- modification of SiO$_2$ NPs reduces the cytotoxicity. Recruitment of neutrophils was the highest by COOH-SiO$_2$ NPs, whereas OH-SiO$_2$ NPs induced the mRNA expression of proinflammatory cytokines to a greater extent than COOH-SiO$_2$ NPs. It is possible that the relatively low recruitment of macrophages and lymphocytes in OH-SiO$_2$ NPs - treated mice resulted from death of these cells, while exposure to OH-SiO$_2$ NPs induced a more extensive inflammatory response, as evident by the higher transcription levels of proinflammatory cytokines in the remaining cells and increased lung weight following exposure to OH-SiO$_2$ NPs.

Flow cytometry showed the uptake of COOH-SiO$_2$ NPs by RAW264.8 macrophages starting from 1 hr after exposure and was higher than those of NH$_2$- and OH-SiO$_2$ NPs. The higher rate of uptake of COOH-SiO$_2$ NPs is in agreement with our recent study, which showed better interaction of COOH-SiO$_2$ NPs with Caco-2 cells compared with NH$_2$- and OH-SiO$_2$ NPs [35].

Confocal microscopy showed localization of OH-SiO$_2$ NPs in the proximity of the cell membrane and less internalization of these particles in RAW264.8 macrophages. This finding suggests that OH-SiO$_2$ NPs-induced cell death of RAW264.8 macrophages is not necessarily through internalization of the particles, but rather the physical contact between the particles and cell membrane, in agreement with the recent finding of the cytolytic toxicity of silanol-rich SiO$_2$ NPs (Spyrogianni et al. 2017).

Increased lung weight can reflect pulmonary inflammation, which is associated with intense protein/fluid leakage from the vasculature into the alveoli mediated by inflammatory signals from pulmonary cells [36]. The results suggest that NH$_2$- and COOH-SiO$_2$ NPs can recruit neutrophils only at the high dose of 10 mg/kg bw, with COOH-SiO$_2$ NPs being more potent in attracting neutrophils, while the lack of dose dependency in increasing the number of neutrophils and the decrease in macrophage numbers with larger dose of OH-SiO$_2$ NPs could be explained by cell death induced by these NPs. With the aim of confirming and further investigating these findings, we analyzed the uptake of fluorescently-labelled SiO$_2$ NPs by neutrophils and macrophages and performed in vitro experiments using RAW264.8 macrophage cell line.

The different patterns of interaction with the cells and subsequent cellular uptake observed in the present study could perhaps explain the differences in the toxicological profile of the three types of SiO$_2$ NPs used in this study. While COOH-SiO$_2$ NPs were efficiently internalized into the cells, OH-SiO$_2$ NPs predominantly interacted with the cell plasma membrane, potentially damaging the membrane and inducing cell death. The zeta potentials of all types of SiO$_2$ NPs in water were negative, in the order of
NH\textsubscript{2}-SiO\textsubscript{2} NPs, OH-SiO\textsubscript{2} NPs and COOH-SiO\textsubscript{2} NPs. On the other hand, the zeta potentials of all types of SiO\textsubscript{2} NPs in the FBS-free media were almost of the same negative values. These results are in agreement with our recent study, which showed greater interaction of Caco-2 cells or THP-1 cells with COOH-SiO\textsubscript{2} NPs than OH- or NH\textsubscript{2}-SiO\textsubscript{2} NPs [35]. The present findings and our previous study show that the difference in the extent of internalization or cytotoxicity is not explained by the differences in zeta potentials. Further studies are needed to understand the mechanism(s) involved in cellular internalization and toxicity of SiO\textsubscript{2} NPs.

**Conclusions**

Carboxyl modification of SiO\textsubscript{2} NPs increases their uptake into macrophages while carboxyl- or amino-modification of SiO\textsubscript{2} NPs reduces cytotoxicity of SiO\textsubscript{2} NPs. The cytotoxicity of OH-SiO\textsubscript{2} NPs could be related to the physical contact between NPs and the cell membrane, though further studies are needed to test this hypothesis and to determine the exact mechanism of SiO\textsubscript{2} NPs-induced cytotoxicity.

**Declarations**

*Ethics approval*

This study was conducted according to the Japanese law on the protection and control of animals and the Animal Experimental Guidelines of Tokyo University of Science. The experimental protocol was also approved by the Animal Ethics Committee of Tokyo University of Science prior to the experiment. The approval number is Y14059.

*Consent for publication*

Not applicable

*Availability of data and materials*

All date generated or analysed during the current study are included in this published article and its supplementary information files.

*Competing interests*

The authors declare that they have no competing interests.

*Funding*

This work was supported by Japan Society for the Promotion of Science (JSPS) International Fellowship for Research in Japan (Short-term Program) #PE13040, JSPS NEXT Program #LS056 and Japan Science and Technology Agency (JST) Strategic Japanese-EU Cooperative Program: Study on managing the potential health and environmental risks of engineered nanomaterials.
Authors’ contribution

SV, SI, SB, LT and GI designed the study and interpreted the data. SV, EW, WW and SI conducted animal studies. EW, KM, ST and YO conducted in vitro studies. CZ, TSa, AS, YH, TSu and RA contributed to acquisition of the data. SV, EW, SI and GI drafted and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank Ms. Kotomi Toriumi, Ms. Haruka Yonekura and Ms. Satoko Arai for the excellent secretarial supports.

References

1. Cheng R, Feng F, Meng F, Deng C, Feijen J, and Zhong Z: Glutathione-responsive nano-vehicles as a promising platform for targeted intracellular drug and gene delivery. J Control Release 2011, 152: 2-12.

2. Huang C, Neoh KG, Wang L, Kang ET, and Shuter B: Surface functionalization of superparamagnetic nanoparticles for the development of highly efficient magnetic resonance probe for macrophages. Contrast Media Mol Imaging 2011, 6: 298-307.

3. Pan J, Wan D, and Gong J: PEGylated liposome coated QDs/mesoporous silica core-shell nanoparticles for molecular imaging. Chem Commun (Cambridge) 2011, 47: 3442-4.

4. Yildirimer L, Thanh NT, Loizidou M, and Seifalian AM: Toxicology and clinical potential of nanoparticles. Nano Today 2011, 6: 585-607.

5. Napierska D, Thomassen LC, Lison D, Martens JA, and Hoet PH: The nanosilica hazard: Another variable entity. Part Fibre Toxicol 2010, 7: 39.

6. Murugadoss S, Lison D, Godderis L, Van Den Brule S, Mast J, Brassinne F, Sebaihi N, and Hoet PH: Toxicology of silica nanoparticles: An update. Arch Toxicol 2017, 91: 2967-3010.

7. Hansen SF, Michelson ES, Kamper A, Borling P, Stuer-Lauridsen F, and Baun A: Categorization framework to aid exposure assessment of nanomaterials in consumer products. Ecotoxicology 2008, 17: 438-47.

8. Kempen PJ, Greasley S, Parker KA, Campbell JL, Chang HY, Jones JR, Sinclair R, Gambhir SS, and Jokerst JV: Theranostic mesoporous silica nanoparticles biodegrade after pro-survival drug delivery and ultrasound/magnetic resonance imaging of stem cells. Theranostics 2015, 5: 631-42.

9. Hnizdo E, Sullivan PA, Bang KM, and Wagner G: Association between chronic obstructive pulmonary disease and employment by industry and occupation in the US population: a study of data from the Third National Health and Nutrition Examination Survey. Am J Epidemiol 2002, 156: 738-46.

10. Ross MH and Murray J: Occupational respiratory disease in mining. Occup Med (Lond) 2004, 54: 304-10.
11. Hnizdo E and Vallyathan V: **Chronic obstructive pulmonary disease due to occupational exposure to silica dust: a review of epidemiological and pathological evidence.** *Occup Environ Med* 2003, 60: 237-43.

12. Yu Y, Duan J, Yu Y, Li Y, Liu X, Zhou X, Ho KF, Tian L, and Sun Z: **Silica nanoparticles induce autophagy and autophagic cell death in HepG2 cells triggered by reactive oxygen species.** *J Hazard Mater* 2014, 270: 176-86.

13. Fedeli C, Selvestrel F, Tavano R, Segat D, Mancin F, and Papini E: **Catastrophic inflammatory death of monocytes and macrophages by overtaking of a critical dose of endocytosed synthetic amorphous silica nanoparticles/serum protein complexes.** *Nanomedicine (Lond)* 2013, 8: 1101-26.

14. Ahmad J, Ahamed M, Akhtar MJ, Alrokayan SA, Siddiqui MA, Musarrat J, and Al-Khedhairy AA: **Apoptosis induction by silica nanoparticles mediated through reactive oxygen species in human liver cell line HepG2.** *Toxicol Appl Pharmacol* 2012, 259: 160-8.

15. Nemmar A, Yuvaraju P, Beegam S, Yasin J, Dhaferi RA, Fahim MA, and Ali BH: **In vitro platelet aggregation and oxidative stress caused by amorphous silica nanoparticles.** *Int J Physiol Pathophysiol Pharmacol* 2015, 7: 27-33.

16. Maser E, Schulz M, Sauer UG, Wiemann M, Ma-Hock L, Wohlleben W, Hartwig A, and Landsiedel R: **In vitro and in vivo genotoxicity investigations of differently sized amorphous SiO2 nanomaterials.** *Mutat Res Genet Toxicol Environ Mutagen* 2015, 794: 57-74.

17. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, and Tschopp J: **Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica.** *Science* 2008, 320: 674-7.

18. Rubio L, Pyrgiotakis G, Beltran-Huarac J, Zhang Y, Gaurav J, Deloid G, Spyrogianni A, Sarosiek KA, Bello D, and Demokritou P: **Safer-by-design flame-sprayed silicon dioxide nanoparticles: the role of silanol content on ROS generation, surface activity and cytotoxicity.** *Part Fibre Toxicol* 2019, 16: 40.

19. Kusaka T, Nakayama M, Nakamura K, Ishimiya M, Furusawa E, and Ogawawara K: **Effect of silica particle size on macrophage inflammatory responses.** *PLoS One* 2014, 9: e92634.

20. Morris AS, Adamcakova-Dodd A, Lehman SE, Wongrakpanich A, Thorne PS, Larsen SC, and Salem AK: **Amine modification of nonporous silica nanoparticles reduces inflammatory response following intratracheal instillation in murine lungs.** *Toxicol Lett* 2016, 241: 207-15.

21. Marzaioli V, Aguilar-Pimentel JA, Weichenmeier I, Luxenhofer G, Wiemann M, Landsiedel R, Wohlleben W, Eiden S, Mempel M, Behrendt H, Schmidt-Weber C, Gutermuth J, and Alessandrini F: **Surface modifications of silica nanoparticles are crucial for their inert versus proinflammatory and immunomodulatory properties.** *Int J Nanomedicine* 2014, 9: 2815-32.

22. Brandenberger C, Rowley NL, Jackson-Humbles DN, Zhang Q, Bramble LA, Lewandowski RP, Wagner JG, Chen W, Kaplan BL, Kaminski NE, Baker GL, Worden RM, and Harkema JR: **Engineered silica nanoparticles act as adjuvants to enhance allergic airway disease in mice.** *Part Fibre Toxicol* 2013, 10: 26.

23. Han B, Guo J, Abrahaley T, Qin L, Wang L, Zheng Y, Li B, Liu D, Yao H, Yang J, Li C, Xi Z, and Yang X: **Adverse effect of nano-silicon dioxide on lung function of rats with or without ovalbumin**
immunization. *PLoS One* 2011, **6**: e17236.

24. Park HJ, Sohn JH, Kim YJ, Park YH, Han H, Park KH, Lee K, Choi H, Um K, Choi IH, Park JW, and Lee JH: **Acute exposure to silica nanoparticles aggravate airway inflammation: Different effects according to surface characteristics.** *Exp Mol Med* 2015, **47**: e173.

25. Han H, Park YH, Park HJ, Lee K, Um K, Park JW, and Lee JH: **Toxic and adjuvant effects of silica nanoparticles on ovalbumin-induced allergic airway inflammation in mice.** *Respir Res* 2016, **17**: 60.

26. Bagwe RP, Hilliard LR, and Tan W: **Surface modification of silica nanoparticles to reduce aggregation and nonspecific binding.** *Langmuir* 2006, **22**: 4357-62.

27. Marzaioli V, Gross CJ, Weichenmeier I, Schmidt-Weber CB, Gutermuth J, Gross O, and Alessandrini F: **Specific surface modifications of silica nanoparticles diminish inflammasome activation and in vivo expression of selected inflammatory genes.** *Nanomaterials (Basel)* 2017, **7**.

28. Lankoff A, Arabski M, Wegierek-Ciuk A, Kruszewski M, Lisowska H, Banasik-Nowak A, Rozga-Wijas K, Wojewodzka M, and Slomkowski S: **Effect of surface modification of silica nanoparticles on toxicity and cellular uptake by human peripheral blood lymphocytes in vitro.** *Nanotoxicology* 2013, **7**: 235-50.

29. Morishige T, Yoshioka Y, Inakura H, Tanabe A, Yao X, Narimatsu S, Monobe Y, Imazawa T, Tsunoda S, Tsutsumi Y, Mukai Y, Okada N, and Nakagawa S: **The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1beta production, ROS production and endosomal rupture.** *Biomaterials* 2010, **31**: 6833-42.

30. Agency EUSEP, *Air Quality Criteria for Particulate Matter Volume II*. 2004, Environmental Protection Agency: Washington, DC.

31. Knust J, Ochs M, Gundersen HJ, and Nyengaard JR: **Stereological estimates of alveolar number and size and capillary length and surface area in mice lungs.** *Anat Rec (Hoboken)* 2009, **292**: 113-22.

32. Fleming TJ, Fleming ML, and Malek TR: **Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family.** *J Immunol* 1993, **151**: 2399-408.

33. Hestdal K, Ruscetti FW, Ihle JN, Jacobsen SE, Dubois CM, Kopp WC, Longo DL, and Keller JR: **Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells.** *J Immunol* 1991, **147**: 22-8.

34. Vranic S, Gosens I, Jacobsen NR, Jensen KA, Bokkers B, Kermanizadeh A, Stone V, Baeza-Squiban A, Cassee FR, Tran L, and Boland S: **Impact of serum as a dispersion agent for in vitro and in vivo toxicological assessments of TiO2 nanoparticles.** *Arch Toxicol* 2017, **91**: 353-363.

35. Tada-Oikawa S, Eguchi M, Yasuda M, Izuoka K, Ikegami A, Vranic S, Boland S, Tran L, Ichihara G, and Ichihara S: **Functionalized surface-charged SiO2 nanoparticles induce pro-inflammatory responses, but are not lethal to Caco-2 cells.** *Chem Res Toxicol* 2020, **33**: 1226-1236.

36. Aman J, van der Heijden M, van Lingen A, Girbes AR, van Nieuw Amerongen GP, van Hinsbergh VW, and Groeneveld AB: **Plasma protein levels are markers of pulmonary vascular permeability and degree of lung injury in critically ill patients with or at risk for acute lung injury/acute respiratory distress syndrome.** *Crit Care Med* 2011, **39**: 89-97.