

Immunotoxicity of silicon dioxide nanoparticles with different sizes and electrostatic charge

Jae-Hyun Kim^{1,*}
 Cheol-Su Kim^{1,*}
 Rosa Mistica Coles Ignacio^{2,*}
 Dong-Heui Kim²
 Ma Easter Joy Sajo²
 Eun Ho Maeng³
 Xu-Feng Qi⁴
 Seong-Eun Park⁵
 Yu-Ri Kim⁷
 Meyoung-Kon Kim⁷
 Kyu-Jae Lee²
 Soo-Ki Kim^{1,5,6}

¹Department of Microbiology, ²Department of Environmental Medical Biology, Wonju College of Medicine, Yonsei University, Wonju-si, Gangwon-do, Republic of Korea;

³Healthcare Laboratory, Medical Device Evaluation, Korea Testing and Research Institute, Wonju-si, Gangwon-do, Republic of Korea; ⁴Key Laboratory for Regenerative Medicine of Ministry of Education and Department of Developmental and Regenerative Biology, School of Life Science and Technology, Ji Nan University, Guangzhou, People's Republic of China;

⁵Institute of Lifestyle Medicine, Wonju College of Medicine, Yonsei University, Wonju-si, Gangwon-do, Republic of Korea; ⁶Institute of Biomaterials, Yonsei University, Wonju Campus, Wonju-si, Gangwon-do, Republic of Korea; ⁷Department of Biochemistry and Molecular Biology, Medical School and College, Korea University, Seoul, Republic of Korea

*These authors contributed equally to this work

Correspondence: Soo-Ki Kim
 Department of Microbiology, Wonju College of Medicine, Yonsei University, Wonju-si, Gangwon-do, 220-701, Republic of Korea
 Tel +82 10 3370 8446
 Fax +82 33 732 4446
 Email kim6@yonsei.ac.kr

Abstract: Silicon dioxide (SiO₂) nanoparticles (NPs) have been widely used in the biomedical field, such as in drug delivery and gene therapy. However, little is known about the biological effects and potential hazards of SiO₂. Herein, the colloidal SiO₂ NPs with two different sizes (20 nm and 100 nm) and different charges (L-arginine modified: SiO₂^{EN20[R]}, SiO₂^{EN100[R]}; and negative: SiO₂^{EN20[-]}, SiO₂^{EN100[-]}) were orally administered (750 mg/kg/day) in female C57BL/6 mice for 14 days. Assessments of immunotoxicity include hematology profiling, reactive oxygen species generation and their antioxidant effect, stimulation assays for B- and T-lymphocytes, the activity of natural killer (NK) cells, and cytokine profiling. In vitro toxicity was also investigated in the RAW 264.7 cell line. When the cellularity of mouse spleen was evaluated, there was an overall decrease in the proliferation of B- and T-cells for all the groups fed with SiO₂ NPs. Specifically, the SiO₂^{EN20(-)} NPs showed the most pronounced reduction. In addition, the nitric oxide production and NK cell activity in SiO₂ NP-fed mice were significantly suppressed. Moreover, there was a decrease in the serum concentration of inflammatory cytokines such as interleukin (IL)-1β, IL-12 (p70), IL-6, tumor necrosis factor-α, and interferon-γ. To elucidate the cytotoxicity mechanism of SiO₂ in vivo, an in vitro study using the RAW 264.7 cell line was performed. Both the size and charge of SiO₂ using murine macrophage RAW 264.7 cells decreased cell viability dose-dependently. Collectively, our data indicate that different sized and charged SiO₂ NPs would cause differential immunotoxicity. Interestingly, the small-sized and negatively charged SiO₂ NPs showed the most potent in vivo immunotoxicity by way of suppressing the proliferation of lymphocytes, depressing the killing activity of NK cells, and decreasing proinflammatory cytokine production, thus leading to immunosuppression.

Keywords: silicon dioxide, nanoparticle, immunotoxicity, oxidative stress, cytokines, immunosuppression

Introduction

The growing fabrication and characterization of silica nanoscale materials has received attention in biomedical research, such as in the development of biosensors, enzyme immobilization, controlled drug release and delivery, and cellular uptake.¹⁻⁶ Since these various studies on nanomedicine have emerged, the medical applications of nanoparticles (NPs) were highlighted, especially for the development of anticancer agents. Mesoporous silica NPs (MSNs) attracted great attention in the last few decades for their wider plausible application in the emerging field of nanomedicine. MSNs are inorganic nanocarriers that are known to be highly stable in a physicochemical and biochemical context; thus, they are vitally important in the construction of anticancer medicine.^{6,7} Recently, these MSNs received United States Food and Drug Administration approval

as inorganic carriers in nanomedicine, and they are considered one of the most promising inorganic nanobiomaterials.^{7,8} Their potential applications seem endless based on the unique physicochemical characteristics of this nanomaterial, such as varying sizes, shapes, chemical composition, and assembly.⁹ Meantime, the special physicochemical characteristics of silica posed concerns about their potential environmental and health implications.¹⁰ To date, animal exposure to colloidal silica confirmed liver damage¹¹ and moderate to severe pulmonary inflammation and tissue damage, primarily induced by oxidative stress and apoptosis.^{12,13} The physicochemical properties play an important role in the toxic reaction of silica NPs. Small particles mean that there is a larger surface area, and this might indicate an increase in surface reactivity, which enables the NPs to interact with cell biomolecules.⁹ The altered surface charge of NPs provides a unique way to facilitate their uptake into the interior structure of the cells.¹⁴ However, most studies have focused on pulmonary and liver toxicity; very few studies have overlooked the toxicological effects of silica NPs on the immune response *in vivo*.^{15–17} In addition, the influence of NP properties (for example, size, surface charge) on their potential hazards to the biological system needs to be elucidated.

In this study, we investigated the potential immunotoxicity of colloidal silicon dioxide (SiO₂) NPs with two different sizes (20 nm and 100 nm) and different charges (L-arginine modified: SiO₂^{EN20[R]}, SiO₂^{EN100[R]}; and negative: SiO₂^{EN20[-]}, SiO₂^{EN100[-]}) in mice and in the RAW 264.7 cell line. Accordingly, cytotoxicity was performed *in vitro* using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). The primary indicators of immune toxicity were also assessed *in vivo*, such as body weight measurement and hematology profiles. The NP-induced oxidative effect was also examined with reactive oxygen species (ROS) generation, superoxide dismutase (SOD) activity, and intracellular levels of glutathione peroxidase (GPx). The cellularity of the spleen and analysis of the functional capacity of specific immune cells were evaluated by stimulation assays for B- and T-lymphocytes, and the activity of natural killer (NK) cells was examined. In addition, we also focused on the inflammatory responses induced by NPs; therefore, the concentration of cytokines was determined.

Materials and methods

Preparation of particle suspensions

Manufactured SiO₂ NPs were purchased from E&B Nanotech Co, Ltd (Ansan, Republic of Korea). The colloidal SiO₂ NPs were already coated with citrate, and they had

these following characteristics: the size and zeta potential of 20 nm (SiO₂^{EN20[-]}) and 100 nm (SiO₂^{EN100[-]}) are 20±2 nm; -19 mV and 90±13 nm; -40 mV, respectively. To shift the strong negative charge of SiO₂ NPs to a positive direction, their surface was modified with L-arginine (R) (SiO₂^{EN20[R]}, SiO₂^{EN100[R]}), as reported.¹⁸ After the surface modification with L-arginine, the size and zeta potential of 20 nm and 100 nm became: 20±2 nm; -9 mV (SiO₂^{EN20[R]}) and 92±9 nm; -22 mV (SiO₂^{EN100[R]}). Then, powdered particle suspensions were prepared by dispersing the particles in 1× phosphate buffered saline (PBS), and to prevent agglomeration and to ensure uniform suspension, particles were sonicated in an ultrasonic bath (Hielscher Ultrasonics GmbH, Teltow, Germany) for 5–10 minutes.

Cell culture

The RAW 264.7 mouse leukemic monocyte macrophage cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's Modified Eagle's Medium (HyClone; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) heat-activated fetal bovine serum (FBS) (HyClone; Thermo Fisher Scientific) and 1% (v/v) antibiotic-antimycotic (Gibco®; Life Technologies) at 37°C in a 5% CO₂ incubator.

Cell viability assay

Approximately 1×10⁵ of RAW 264.7 cells were seeded into each well of 96-well plates. After reaching 70% confluence, the culture medium in the plate was replaced with a freshly prepared 100 µL of SiO₂ NP suspension, which was diluted to appropriate concentrations (10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, 160 µg/mL, 320 µg/mL, and 640 µg/mL) with the culture medium, and the cells were incubated for 24 hours at 37°C in a 5% CO₂ incubator. Cells not treated with SiO₂ NPs served as a control in the experiment. Afterwards, a commercially available CCK-8 was used to evaluate the cytotoxic effect of SiO₂ NPs. Then, 10 µL of the CCK-8 solution was added to each well and incubated at 37°C for 1 hour. Colored supernatants were measured at 450 nm by a DTX-880 multimode microplate reader (Beckman Coulter, Inc., Brea, CA, USA). All experiments were performed in triplicate.

Animals and exposure

For this experiment, 6-week-old female C57BL/6 (number [n]=5) mice weighing 18±2 g were purchased from Orient Bio Inc. (Seongnam, Republic of Korea) and were maintained at 22°C±2°C and 40%–60% humidity under a 12:12 hours

light–dark cycle. The mice were acclimatized for 1 week and randomly assigned to one of five groups: normal control (purified water alone); or one of four experimental groups treated with four types of SiO₂ NPs (SiO₂^{EN20[R]}, SiO₂^{EN100[R]}, SiO₂^{EN20[-]}, and SiO₂^{EN100[-]}), respectively. All of the animals were orally administered 750 mg/kg of an SiO₂ NP suspension daily for 2 continuous weeks. During the administration periods, the animals' body weight was recorded. The animal use protocol for this experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Wonju College of Medicine, Yonsei University Wonju Campus (Wonju-si, Gangwon-do, Republic of Korea).

Analysis of blood samples

Animals were sacrificed following the 2-week oral administration of SiO₂ NPs. Their blood was collected from the retro-orbital venous plexus, and the total white blood cell (WBC) count was collected in tubes coated with an anticoagulant. The blood was mixed with an automatic mixer for 5 minutes, and the WBCs and their differential counts (such as neutrophils, lymphocytes, monocytes, eosinophils, and basophils) were determined by an automatic blood analyzer (Hemavel[®] HV950 FS; Drew Scientific, Erba Diagnostics, Inc., Dallas, Texas, USA).

Preparation of splenocytes

For the isolation of splenocytes, spleens were aseptically removed from recently sacrificed mice and the tissue was transferred to a tube containing 1× PBS on ice. Splenocyte suspensions were prepared by blandly squeezing the spleen between the frosted ends of the two sterile microscope slides into a 100 mm tissue culture grade Petri dish. The slides were rinsed at regular intervals with 1× PBS. The cells were formed into a single suspension using a pipette. After that, the cell suspensions were filtered through a cell strainer. Next, the cell suspensions were centrifuged at 1,500 rpm at 4°C for 5 minutes to produce pellets. For the optimal lysis of erythrocytes, the pellets were resuspended in 5 mL of red blood cell lysis buffer and incubated on ice for 5 minutes with occasional shaking. The reaction was stopped by diluting the lysis buffer with 25 mL of 1× PBS. Thereafter, cells were spun (1,500 rpm at 4°C for 5 minutes), and the supernatant was carefully removed. The pellet was then washed two times in 1× PBS and resuspended in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 3% FBS and 1% antibiotic–antimycotic. Cells were then counted. The viability of the cells used in all the experiments was higher than 95%, as measured

by the trypan blue exclusion method (Sigma-Aldrich, St Louis, MO, USA).

Immune cell proliferation

Single cell suspensions of lymphocytes from the spleen were prepared. Splenocytes were counted in a hemocytometer, and 1×10⁵ cells per well were suspended in a 96-well flat-bottom plate in 100 μL of RPMI-1640 supplemented with 10% heat-activated FBS and 1% antibiotics. Thereafter, cells were treated with 2.5 mg/mL of Concanavalin A (Con-A) (Sigma-Aldrich) and 1 mg/mL lipopolysaccharide (LPS) (Sigma-Aldrich) with 1 μL/well. The treated cells were incubated at 37°C in a humidified atmosphere under 5% CO₂ for 2 hours. Cell proliferation was determined using a CCK-8 kit according to the manufacturer's instructions. Absorbance was measured at 450 nm by a DTX-880 multi-mode microplate reader.

NK cytotoxicity assay

The murine lymphoma cancer cell line, YAC-1 (American Type Culture Collection), as target cells were cocultured with NK-enriched murine splenocytes. The percentage of target cells killed by effector NK cells was determined. Briefly, various effector cell dilutions were prepared: 1×10⁵ cells/well for 100:1; 5×10⁴ cells/well for 50:1; and 2.5×10⁴ cells/well for 25:1. The target cells (1×10³ cells/well) were then cocultured with the different effector cell dilutions prepared in a 96-well plate and incubated for 6 hours at 37°C in a humidified incubator with 5% CO₂. The CCK-8 was conducted according to the manufacturer's instructions. A special highly water soluble tetrazolium salt (WST-8) (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5[2-4disulfophenyl]-2H-tetrazolium, monosodium salt) was added to the culture. WST-8 is reduced by dehydrogenase activities in cells to give the orange formazan dye, which is soluble in the culture media. The amount of the formazan dye generated by dehydrogenase in cells is directly proportional to the number of living cells. After 1 hour of incubation with the WST-8 solution, the cell suspension was then colorimetrically measured at 450 nm by a DTX-880 multimode microplate reader, and the number of live cells at the different ratios was determined.

Measurement of nitric oxide (NO) production

The nitrite (NO₂⁻) present in the supernatant of splenocytes was used as an indicator of nitric oxide (NO) production using the Griess reagent (Promega Corporation, Fitchburg,

WI, USA). The assay relies on measuring nitrite (NO_2^-), one of the primary, stable, and nonvolatile breakdown products of NO. Briefly, 50 μL of the splenocyte supernatant was mixed with an equal volume of Griess reagent in a 96-well microtiter plate and incubated at room temperature for 15 minutes. The absorbance was read at 540 nm using a DTX-880 multimode microplate reader. The NO_2^- concentration was calculated by comparison with the representative NO_2^- standard curve generated by serial two-fold dilutions of sodium nitrate.

Intracellular ROS detection

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect the intracellular ROS level. DCFH-DA can enter into cells as a fluorescent probe, and be acetylated to form DCFH (2',7' dichlorofluorescein) trapped in the cells. The oxidation of DCFH (nonfluorescent) by the intracellular ROS transforms it to DCF (dichlorofluorescein) (fluorescent). The fluorescent intensity of DCF can indicate intracellular ROS quantity. The spleens from mice were collected, and splenocytes were prepared as described in the "Preparation of splenocytes" section. Splenocytes (1×10^6 cells) were incubated with 10 $\mu\text{mol/L}$ of DCFH-DA for 30 minutes at 37°C in the dark. After washing twice with PBS, the splenocytes were analyzed by a DTX-880 multimode microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Antioxidant endogenous enzyme activities

The activity of SOD and GPx were measured using a BioVision kit (BioVision, Inc., Milpitas, CA, USA). Splenocyte suspensions were lysed in ice-cold radioimmunoprecipitation assay buffer, the crude cell lysate was centrifuged at 14,000 rpm for 5 minutes at 4°C , and the cell debris was discarded. The supernatant was then checked for protein concentration using the Bradford method.¹⁹ Thereafter, supernatant was used to measure the activities of different antioxidant enzymes (SOD, GPx) according to the manufacturer's instructions (BioVision, Inc., Milpitas, CA, USA).

Multiplex cytokine analysis

Serum levels of selected cytokines, such as interleukin (IL)-1 β , IL-6, IL-10, IL-12p70, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ , were measured using a Luminex bead-based suspension array system (Bio-Rad Laboratories, Hercules, CA, USA). This Luminex-based multiplexed system combines the principle of a sandwich immunoassay with fluorescent bead-based technology.

Briefly, each set of premixed beads coated with the target antibodies was added to the well, and it was incubated with the sample in a 96-well round-bottomed microtiter plate to react with specific analytes. Then, premixed detection antibodies were added to the wells followed by a fluorescently labeled reporter molecule that specifically binds the analyte. Standard curves for each cytokine were generated using the standard control concentrations provided in the kit. Each step requires a specific incubation time, with shaking at room temperature and washing steps. All washes were performed using a Bio-Plex[®] Pro wash station (Bio-Rad Laboratories). Finally, the samples were then read using the Bio-Plex[®] suspension array reader, and the raw fluorescence data were analyzed using the Bio-Plex[®] Manager[™] software using five-parameter logistic fitting (Bio-Rad Laboratories).

Statistical analysis

For the cell viability assay, the half maximal effective concentration (EC_{50}) was calculated via nonlinear regression using GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). For the remaining in vivo analysis, data values were expressed as the mean \pm standard deviation. The mean values among the groups were analyzed and compared using one-way analysis of variance followed by a subsequent multiple comparison test (Tukey) with the GraphPad Prism version 5.0 software package. Differences were considered statistically significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

Cytotoxicity assay

After exposure for 24 hours at varying doses of SiO_2 , murine macrophage RAW 264.7 cell viability detected by the CCK-8 assay resulted in an explicit dose-dependent reduction (Figure 1). Also, size- and electrostatic charge-dependent cytotoxicity of SiO_2 NPs were found. The EC_{50} values after 24 hours of exposure were 71.10 $\mu\text{g/mL}$ ($\text{SiO}_2^{\text{EN20}(-)}$), 211.4 $\mu\text{g/mL}$ ($\text{SiO}_2^{\text{EN100}(-)}$), 233.2 $\mu\text{g/mL}$ ($\text{SiO}_2^{\text{EN20}(\text{R})}$), and 833.6 $\mu\text{g/mL}$ ($\text{SiO}_2^{\text{EN100}(\text{R})}$).

Body weight measurement

Body weight gain or loss may indicate an indirect immunotoxic effect. To evaluate the toxicity of SiO_2 NPs, mice were fed 750 mg/kg of SiO_2 every day for 2 weeks, and their body weight condition was monitored every 5 days for 2 weeks. After 2 weeks of feeding with SiO_2 NPs, the groups $\text{SiO}_2^{\text{EN20}(-)}$ and $\text{SiO}_2^{\text{EN100}(\text{R})}$ showed significant elevated body weight ($P < 0.05$) compared to the nontreated group (NC). However,

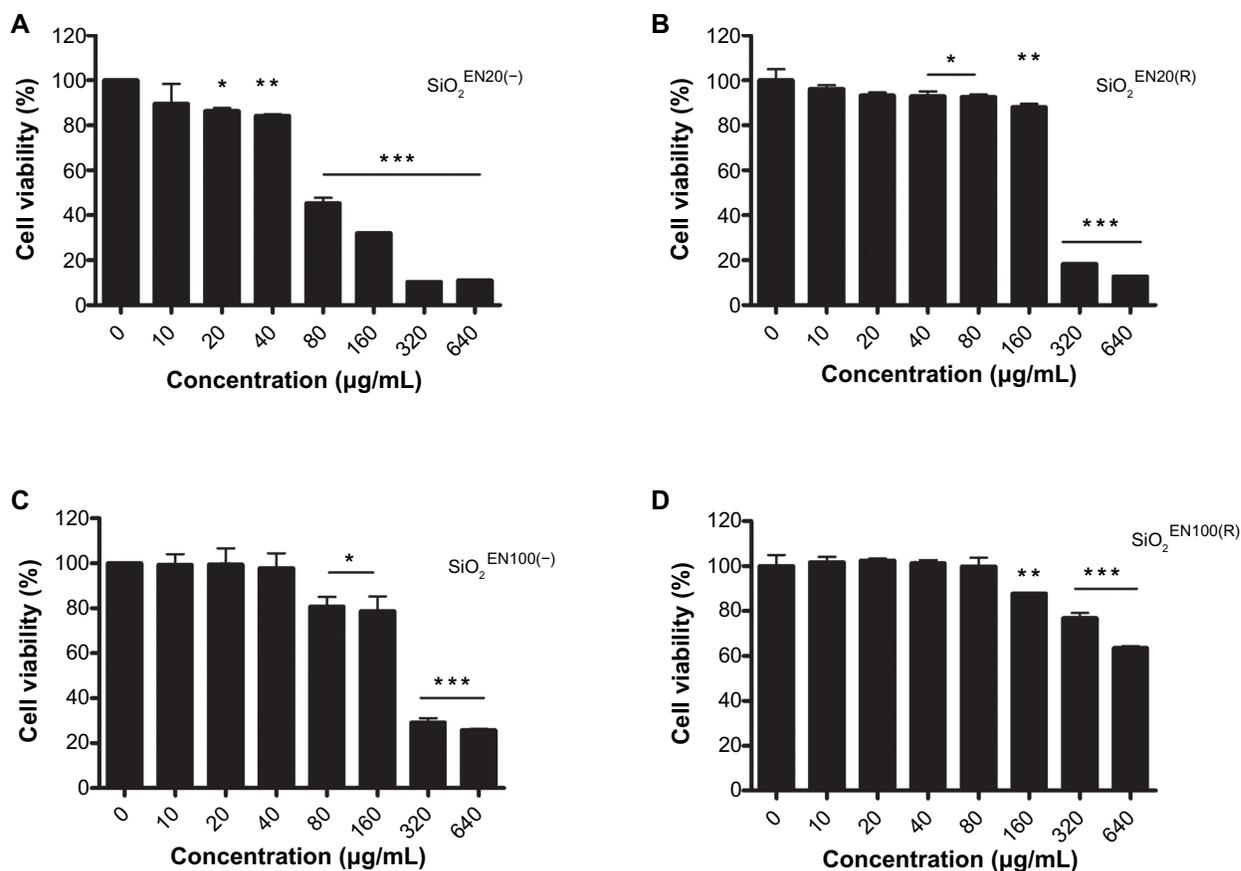


Figure 1 Effect of differently sized and electrostatic charged SiO₂ NPs on the viability of murine macrophage RAW 264.7 cells.

Notes: Cells were incubated with indicated concentrations of colloidal SiO₂ NPs: (A) SiO₂^{EN20(-)}; (B) SiO₂^{EN20(R)}; (C) SiO₂^{EN100(-)}; and (D) SiO₂^{EN100(R)} for 24 hours. Cells not treated with SiO₂ NPs served as the control in the experiment. A commercially available cell viability assay Cell Counting Kit-8 was used to evaluate the cytotoxicity effect of the SiO₂ NPs according to manufacturer's instructions (Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Data are presented as the mean ± standard deviation. **P*<0.05; ***P*<0.01; ****P*<0.001 versus control (0 µg/mL).

Abbreviations: SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; SiO₂, silicon dioxide; NPs, nanoparticles.

the SiO₂^{EN20(R)}-fed group showed significant depressions in body weight (*P*<0.05) compared to the NC. Further, the SiO₂^{EN100(-)}-fed group showed no obvious difference in body weight compared to the NC (Figure 2).

White blood cells (WBCs) and their differential count

Total WBC counts are used to evaluate the severity of inflammation. The altered WBC counts may be indicative of direct or indirect effects of the NPs on cellular proliferation. The WBCs of mice fed orally with SiO₂^{EN100(-)} for 14 days showed depressed counts, which were most prominent on the differential count of lymphocytes (Table 2). Further, the SiO₂^{EN20(-)}-fed group showed slight decreases in WBC count and each type of WBC: lymphocytes and eosinophils. Moreover, the SiO₂^{EN100(R)}-fed group showed no obvious change in WBC count compared to the NC group (Table 1). However, the SiO₂^{EN20(R)}-fed group showed an elevated WBC count for each type of WBC.

Stimulation assays for B- and T- cell lymphocytes

All immune responses are mediated by lymphocytes.²⁰ There are two main classes of lymphocytes: B-lymphocytes (B-cells) and T-lymphocytes (T-cells). Con-A and bacterial LPS are known to preferentially stimulate T-lymphocytes and B-lymphocytes, respectively. Stimulation assays performed on spleen-cell suspensions have been used as immune toxicity indicators. Splenocytes of mice treated with SiO₂^{EN20(-)} showed significantly reduced B-cell (*P*<0.01) and slightly reduced T-cell counts (Figure 3A and B). SiO₂^{EN20(R)} and SiO₂^{EN100(R)} also showed significant reductions in B-cell (*P*<0.001) and T-cell counts (*P*<0.05; *P*<0.001, respectively). On the other hand, splenocytes from SiO₂^{EN100(-)} mice showed significantly elevated B-cell (*P*<0.05) and T-cell counts (*P*<0.001) (Figure 3A and B).

NO production

NO is a reactive molecule that reacts with ROS to produce reactive nitrogen species; moreover, it is recognized as a

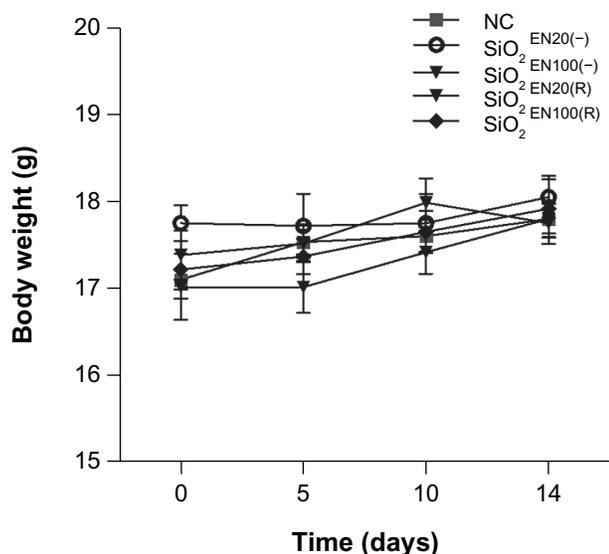


Figure 2 The effect on body weight of SiO₂ NPs fed to mice.

Notes: C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges (SiO₂^{EN(R)}; negative, SiO₂^{EN(-)}) of 750 mg/kg colloidal SiO₂ NPs for 14 days. Body weight was measured all throughout the experiment period, and it served as one of the primary indicators of SiO₂ NP immune toxicity. Data are presented as the mean ± standard deviation; n=5.

Abbreviations: NC, normal control; SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; SiO₂, silicon dioxide; NP, nanoparticle; n, number.

mediator and regulator of immune responses.²¹ NO has various physiological and pathophysiological responses, depending on its relative concentration.²² Here, we measured the level of NO after exposure to SiO₂ NPs. Our results showed that both SiO₂^{EN20(-)} and SiO₂^{EN100(-)} showed significant decreases in NO production compared to the NC (Figure 4A). However, there was no substantial difference in the NO level in SiO₂^{EN20(R)} when compared to the NC group.

ROS production and enzyme activities

Metal oxides mediate toxicity through oxidative stress.²³ The potential of SiO₂ NPs, as well as the influence of NP size and charge, in inducing oxidative stress was examined by measuring the ROS generation and the antioxidant activity

Table 1 Total WBC and type of WBC counts in L-arginine surface modified SiO₂ NP-fed mice

WBC and members	NC	SiO ₂ ^{EN20(R)}	SiO ₂ ^{EN100(R)}
Total WBC, ×10 ⁹ /L	4.030±0.600	4.843±1.525	4.017±1.452
Neutrophil, ×10 ⁹ /L	0.673±0.129	0.828±0.362	0.568±0.191
Lymphocyte, ×10 ⁹ /L	3.258±0.565	3.800±1.127	3.270±1.190
Monocyte, ×10 ⁹ /L	0.092±0.025	0.160±0.072	0.162±0.087
Eosinophil, ×10 ⁹ /L	0.003±0.005	0.040±0.083	0.003±0.005
Basophil, ×10 ⁹ /L	0.002±0.004	0.015±0.023	0.002±0.004

Notes: Data are the mean ± standard deviation; n=5.

Abbreviations: WBC, white blood cell; SiO₂, silicon dioxide; NP, nanoparticle; NC, normal control; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; n, number.

Table 2 Total WBC and type of WBC counts in negatively charged SiO₂ NP-fed mice

WBC and members	NC	SiO ₂ ^{EN20(-)}	SiO ₂ ^{EN100(-)}
Total WBC, ×10 ⁹ /L	6.047±0.797	5.623±0.737	5.087±1.423
Neutrophil, ×10 ⁹ /L	0.762±0.247	0.690±0.220	0.775±0.327
Lymphocyte, ×10 ⁹ /L	5.060±0.721	4.768±0.526	4.138±1.057
Monocyte, ×10 ⁹ /L	0.172±0.056	0.153±0.041	0.137±0.031
Eosinophil, ×10 ⁹ /L	0.038±0.041	0.010±0.011	0.025±0.052
Basophil, ×10 ⁹ /L	0.010±0.011	0.000±0.000	0.008±0.012

Notes: Data are the mean ± standard deviation; n=5.

Abbreviations: WBC, white blood cell; SiO₂, silicon dioxide; NP, nanoparticle; NC, normal control; SiO₂^{EN(-)}, negatively charged silicon dioxide.

of SOD and GPx levels in mice. We found that SiO₂^{EN20(-)} and SiO₂^{EN100(-)}-fed mice showed a slight decrease in ROS production, while SiO₂^{EN20(R)}- and SiO₂^{EN100(R)}-fed mice showed increased production of ROS compared to the NC group (Figure 4B). Furthermore, both the size and electrostatic charge of SiO₂ had no effect on intracellular activities of SOD (Figure 5A), and they had little effect on GPx (Figure 5B). There was a slight increase in the activity of SiO₂^{EN20(R)}- and SiO₂^{EN100(R)}-fed mice, which can be correlated to the slight decrease in ROS production among these groups.

NK cell activity

NK cells are activated in response to cytokine production.²⁴ NK cells were incubated with target cells (YAC-1) at different ratios. In particular, SiO₂^{EN20(R)} and SiO₂^{EN20(-)} showed the most significant reduction ($P<0.001$) in the killing activity of the effector cell (NK), while SiO₂^{EN100(R)} and SiO₂^{EN100(-)} showed comparable activity with the NC group (Table 3).

Cytokine analysis

Exposure to NPs can affect the production of inflammatory and T helper (Th)1-type cytokines. Varying the size and surface charge of SiO₂ NPs differentially influenced cytokine production in the serum. Exposure with SiO₂^{EN20(-)}, SiO₂^{EN100(-)}, and SiO₂^{EN100(R)} led to suppressed production of cytokines, namely IL-1β, TNF-α, IL-12p70, IL-6, and IFN-γ (Table 4). Conversely, SiO₂^{EN20(R)} showed slight increases in IFN-γ. In addition, there were unaltered levels of Th1 cytokine (IL-12p70) and proinflammatory cytokine (TNF-α) among the group exposed to SiO₂^{EN20(R)}, which was in reference to the NC group (Table 4).

Discussion

There is an almost unanimous opinion about the potential toxicity of NP exposure, and it includes ROS generation, proinflammatory responses, and cell death.^{25,26} However,

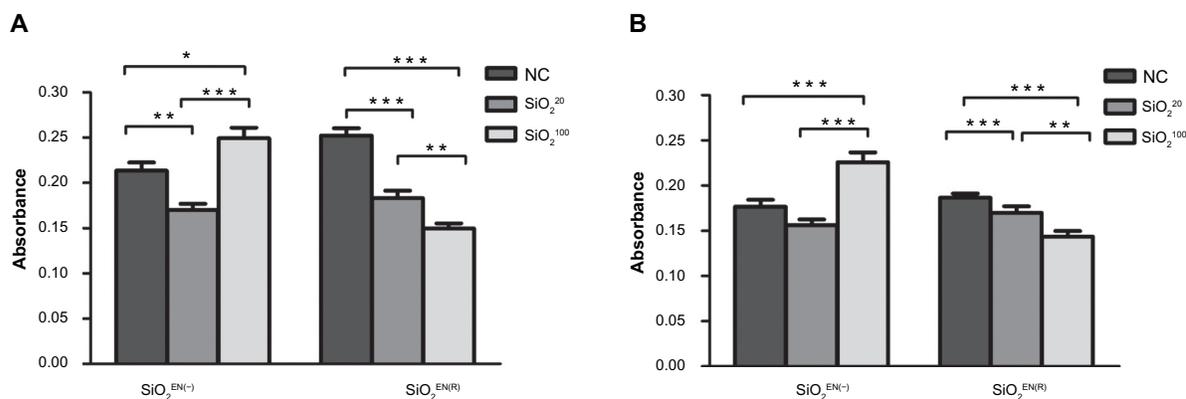


Figure 3 The effect on primary lymphocyte proliferation of SiO₂ NPs fed to mice.

Notes: C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges (SiO₂^{EN(R)}; negative, SiO₂^{EN(-)}) of 750 mg/kg colloidal SiO₂ NPs for 14 days. At the end of the treatment period, single-cell suspensions were made from spleens of mice, and the proliferation of (A) B-cells and (B) T-cells were assessed. Data are presented as the mean ± standard deviation; n=5. *P<0.05, **P<0.01, and ***P<0.001 indicate significant differences when tested with ANOVA. Tukey's test was used for post hoc tests.

Abbreviations: NC, normal control; SiO₂, silicon dioxide; SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; NP, nanoparticle; n, number; ANOVA, analysis of variance.

most of the studies on NPs have focused on lung and liver toxicity, such that the toxic effect of NPs on the immune system is poorly documented.^{15,16} In addition, the influence of NP size and electrostatic charge on potential immunotoxicity remains to be elucidated. The present study addressed the immunotoxicity of different sizes and electrostatic charges of SiO₂ NPs that were fed to mice for 14 days (orally administered at doses of 750 mg/kg/day). In particular, our study shows that immune dysfunction from exposure to the varying sizes and electrostatic charges of SiO₂ would lead to immunosuppression. This was evidenced by suppressing the proliferation of lymphocytes, by depressing the killing activity of NK cells, and by decreasing inflammatory cytokine production. In addition, our data showed that different sizes and charges of SiO₂ NPs could lead to differential immu-

notoxicity in vivo. To clarify this, we first investigated the immune function in mouse spleen lymphocytes. Mitogens such as Con-A and LPS are used to stimulate T-cells and B-cells, respectively, and to assess immune function. We found that the proliferations of B- and T-cells were reduced when mice were fed with SiO₂ NPs (SiO₂^{EN20(R)}, SiO₂^{EN20(-)}, and SiO₂^{EN100(-)}). This is parallel to the decreased lymphocyte count in the blood, as shown in Table 1 and Table 2. Our results are similar to the findings of Lee et al,²⁷ who demonstrated that the intraperitoneally administered 100 nm colloidal silica NPs showed no increase in their proliferative responses to the lymphocyte mitogens. Since lymphocytes play pivotal roles in the immune response, altered B- and T-cell proliferations might culminate in the dysregulation of the immune response.²⁸ This result might suggest that SiO₂

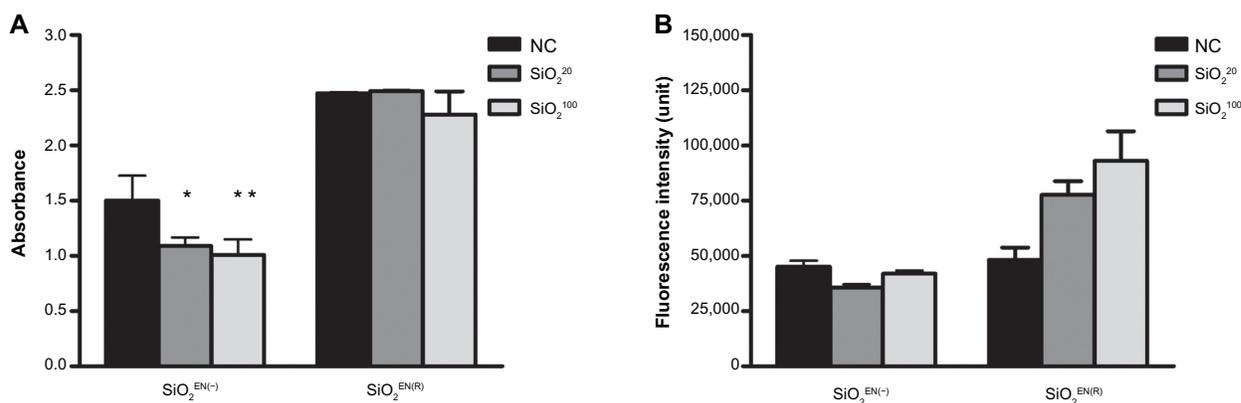


Figure 4 The effect on NO and ROS production of SiO₂ NPs fed to mice.

Notes: C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges (SiO₂^{EN(R)}; negative, SiO₂^{EN(-)}) of 750 mg/kg colloidal SiO₂ NPs for 14 days. The supernatant of splenocytes from mice were harvested and (A) NO₂⁻ accumulation was assessed using the Griess colorimetric assay according to manufacturer's instructions (Promega Corporation, Fitchburg, WI, USA), and (B) intracellular ROS accumulation was detected by fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate using a fluorescence microplate reader. Data are presented as the mean ± standard deviation; n=5. *P<0.05, and **P<0.01 indicate significant differences when tested with ANOVA. Tukey's test was used for post hoc tests.

Abbreviations: NC, normal control; SiO₂, silicon dioxide; SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; NP, nanoparticle; NO, nitric oxide; ROS, reactive oxygen species; NO₂⁻, nitrate; n, number; ANOVA, analysis of variance.

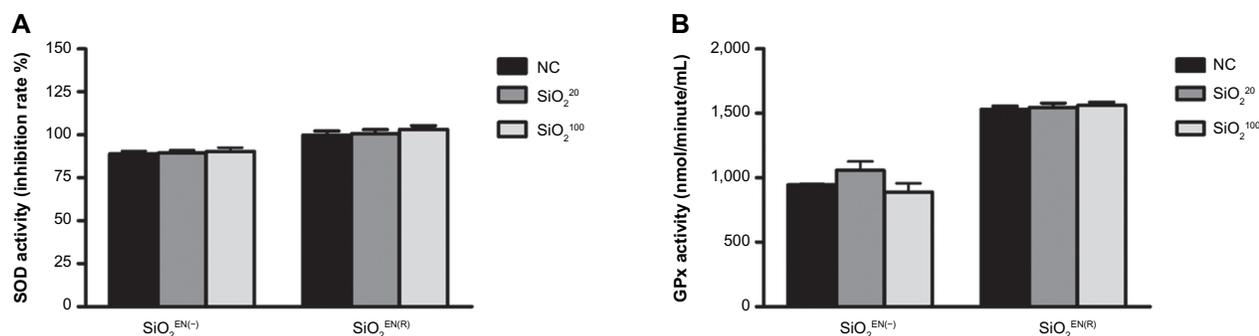


Figure 5 The effect on antioxidant activity of SiO₂ NPs fed to mice.

Notes: C57BL/6 mice were treated with varying sizes (20 nm, 100 nm) and charges (SiO₂^{EN(R)}; negative, SiO₂^{EN(-)}) of 750 mg/kg colloidal SiO₂ NPs for 14 days. Splens from mice were isolated aseptically and made into single-cell suspension splenocytes. (A) SOD activity and (B) GPx activity were measured in splenocyte lysate by an SOD activity assay kit and by a GPx activity colorimetric assay kit, according to the manufacturer's instructions (BioVision, Inc., Milpitas, CA, USA). Data are presented as the mean ± standard deviation; n=5.

Abbreviations: SOD, superoxide dismutase; NC, normal control; SiO₂, silicon dioxide; SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; GPx, glutathione peroxidase; n, number.

NPs, specifically the groups fed negatively charged NPs, may show a decrease in the white pulp component of the spleen, as there is a significant decrease in lymphocyte count.

Cumulative data showed that oxidative stress is another toxic mechanism of NPs.^{17,29} Oxidative stress and NO are closely linked to inflammatory responses. NO is implicated in phagocytosis, as well as in the pathogenesis of inflammation.^{30,37} The impaired production of NO leads to undesired effects, such as inflammation and tissue damage.³⁰ Thus, the abnormal production of NO, such as increases or decreases upon exposure to silica NPs, may induce a proinflammatory response.³⁰ ROS is known to stimulate NO production. Of note, there is the decreased generation of ROS in negatively charged SiO₂^{EN20(-)} and SiO₂^{EN100(-)}-fed mice; therefore, there was decreased NO generation in these given groups. Further, increased production of ROS in SiO₂^{EN20(R)}- and SiO₂^{EN100(R)}-fed mice led to increased NO generation, as compared to the SiO₂^{EN20(-)}- and SiO₂^{EN100(-)}-fed groups. The NP surface charge influenced the capacity of ROS production.^{31,32} L-arginine-coated SiO₂ NPs are capable of inducing intracellular ROS; however, the decreased ROS production among the mice that were fed negatively charged SiO₂ NPs might be due to earlier or later ROS production that occurred in these groups. In

addition, the decreased ROS generation among mice fed negatively charged SiO₂^{EN20(-)} and SiO₂^{EN100(-)} may be due to the leakage of the fluorescent product from the cell due to membrane damage, as there is a higher tendency of cytotoxicity on negative charge, as shown by in vitro viability. Size and charge influence the cytotoxicity of engineered nanomaterials.^{25,32} When comparing the cytotoxicity of particle size and surface charge, we found that negatively charged NPs tended to be higher in toxicity, and 20 nm NPs were the most toxic in a murine macrophage cell line (RAW 264.7) (see Figure 1). In line with our results, the reported data showed that phagocytic cells preferentially interacted with negatively charged particles.³³ Therefore, the higher toxicity of the negatively charged SiO₂^{EN20(-)} is due, in part, to the stronger interaction with the macrophage cells. Given this, the in vitro cytotoxicity and in vivo ROS production showed that negatively charged SiO₂ NPs are more toxic than their negative counterparts. To further prove our findings, we selected other immunotoxicity parameters, such as cytokine profiling and NK cell killing activity.

Any invading pathogens can trigger an inflammatory response, which involves the secretion of inflammation-induced mediators such as cytokines.³⁴ In an inflammatory response, activated immune cells recognize the NPs by their

Table 3 NK cell activity in SiO₂ NP-fed mice

Effector/target ratio	NK cell activity (% mean ± SD)				
	NC	SiO ₂ ^{EN20(-)}	SiO ₂ ^{EN100(-)}	SiO ₂ ^{EN20(R)}	SiO ₂ ^{EN100(R)}
100:1	76.61±3.622	46.95±6.117*	71.12±19.99	55.04±4.845**	82.50±4.560
50:1	58.11±1.691	40.45±3.662	48.87±10.36	45.39±1.263	58.96±3.361
25:1	53.12±2.229	37.51±1.1160	42.91±2.325	46.07±0.842	54.71±2.899

Notes: Data are presented as the mean ± SD; n=5. Nonparametric Tukey's multiple comparison test (*P<0.01; **P<0.001 versus NC).

Abbreviations: NK, natural killer; SiO₂, silicon dioxide; NP, nanoparticle; SD, standard deviation; NC, normal control; SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; n, number.

Table 4 Serum cytokine concentration in mice exposed to SiO₂

Cytokine	NC	SiO ₂ ^{EN20(R)}	SiO ₂ ^{EN100(R)}	SiO ₂ ^{EN20(-)}	SiO ₂ ^{EN100(-)}
IL-1β	473.54±277.32	279.72±130.19	255.95±130.19	287.81±260.52	220.30±118.85
IL-6	1.798±1.111	1.515±0.408	1.265±0.187	1.905±2.242	1.315±0.358
IL-10	41.993±15.239	42.192±6.610	30.587±8.558	38.268±5.874	36.087±7.070
IL-12p70	13.880±3.625	13.148±3.848	9.062±2.948*	6.702±1.997**	6.713±1.369**
IFN-γ	0.747±0.324	0.930±0.241	0.692±0.176	0.508±0.146	0.655±0.167
TNF-α	122.54±54.341	120.60±52.055	75.060±23.715	102.57±64.786	46.717±21.941*

Notes: Data are presented as the mean ± standard deviation; n=5. Nonparametric Tukey's multiple comparison test (*P<0.05; **P<0.001 versus NC).

Abbreviations: SiO₂, silicon dioxide; NC, normal control; SiO₂^{EN(-)}, negatively charged silicon dioxide; SiO₂^{EN(R)}, silicon dioxide modified with L-arginine; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; n, number.

unique physicochemical characteristics, such as their surface charge and surface properties, thus inducing the cytokines to attract more cells to eradicate the NPs.²⁰ Electrostatic charges of the NPs are important parameters in an inflammatory response. As noted by Tan et al,³⁵ cationic (positively charged) engineered nanomaterials, such as liposomes, induced the secretion of cytokines, such as TNF, IL-12, and IFN-γ. In relation to this, mice fed L-arginine surface modified NPs (SiO₂^{EN20(R)} and SiO₂^{EN100(R)}) showed induced secretion of cytokines such as IL-12, IFN-γ, and TNF-α, as compared to the anionic NP (negatively charged SiO₂^{EN20(-)} and SiO₂^{EN100(-)})-fed mice. Consistent with these results, the SiO₂^{EN20(R)}-fed group showed increased WBC production, as compared to the negatively charged (SiO₂^{EN20(-)} and SiO₂^{EN100(-)})-fed mice (which are both in reference to their corresponding normal control group WBC levels). With respect to the relationship between the WBC level and each type of WBC (such as lymphocytes and monocytes) with inflammation, activated WBCs trigger the secretion of cytokines.³⁶ Schwentker et al³⁷ reported that NO could affect the expression and activity of cytokines. Further, particle size and surface area are also important parameters that affect in vivo bioreactivity.³⁸ NPs are predictive in stimulating cytokine production, specifically the ultrafine particles.³⁹ Our study showed that induction of cytokine production is clearly seen in SiO₂^{EN20(R)}-fed mice. NO production was low in negatively charged SiO₂^{EN20(-)}-fed mice. In parallel, the secretion of cytokines (IL-1β, TNF-α, IL-12p70, and IFN-γ) in negatively charged SiO₂^{EN20(-)} and SiO₂^{EN100(-)}-fed mice was repressed. Of these cytokines, IL-12p70 (SiO₂^{EN20(-)}, SiO₂^{EN100(-)}) and TNF-α (SiO₂^{EN100(-)}) were significantly decreased in concentration, as compared to the levels found among the SiO₂^{EN20(R)} and SiO₂^{EN100(R)}-fed mice and among the mice in the NC group. Negatively charged SiO₂^{EN20(-)}-fed mice showed the least secretion of IFN-γ among all of the groups. These two cytokines (IL-12p70 and IFN-γ) are involved in the activation of NK cells, which were found in decreased

concentrations in mice fed with negatively charged SiO₂^{EN20(-)} NPs. In addition, the decreased proliferation of B-cells in negatively charged SiO₂^{EN20(-)}-fed mice also supports our findings, where B-cells also function to secrete cytokines such as IL-1, IL-10, IL-6, IFN-γ, and TNF-α which, in turn, activates the antigen-presenting cells.⁴⁰ Physicochemical properties such as the size and charge of NPs determine their immunotoxicity, and they may also enhance their biological reactivity.⁴¹ The biological activity of NPs increases as the particle size decreases.⁴² However, this trend can be altered by a small change in the particle's surface charge. NPs with positively charged surfaces could be more easily up-taken due to the attractive interaction to the negative cell membrane.⁴³ Despite using varying sizes and electrostatic charges of NPs in this study, other factors need to be considered as well, such as the different exposure periods (chronic or acute) and the different dosages (low, medium, and high) of the NPs, in order to fully discuss, in detail, the potential immunotoxicity of SiO₂. In addition, our study focused on the spleen, as it was suggested by other studies that the spleen is one of the major target organs for toxicity.^{44,45} Moreover, the spleen is also involved in the initiation of immune responses, such that lymphocyte proliferation make take place in this given organ.²⁷ However, to fully elucidate the biosafety of SiO₂ NPs in particular, the immune system and other target organs, such as the lymph nodes and liver, need to be tested. While this paper provides evidence on SiO₂ immunotoxicity, the underlying mechanism still needs to be elucidated.

Conclusion

Collectively, our data indicate that different sized and charged SiO₂ NPs would cause differential in vivo immunotoxicity. Interestingly, the mice fed with the negatively charged SiO₂ NPs exhibited higher cytotoxicity and in vivo immunotoxicity than their L-arginine modified charged counterparts by way of suppressing the proliferation of the lymphocytes, depressing the killing activity of NK cells, and decreasing inflammatory

cytokine production, thus leading to immunosuppression. This is the first account of immunosuppression of differently sized and charged SiO₂ NPs.

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Disclosure

The authors report no conflicts of interest in this work.

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