

# Immunotoxicity of zinc oxide nanoparticles with different size and electrostatic charge

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**Abstract:** While zinc oxide (ZnO) nanoparticles (NPs) have been recognized to have promising applications in biomedicine, their immunotoxicity has been inconsistent and even contradictory. To address this issue, we investigated whether ZnO NPs with different size (20 or 100 nm) and electrostatic charge (positive or negative) would cause immunotoxicity in vitro and in vivo, and explored their underlying molecular mechanism. Using Raw 264.7 cell line, we examined the immunotoxicity mechanism of ZnO NPs as cell viability. We found that in a cell viability assay, ZnO NPs with different size and charge could induce differential cytotoxicity to Raw 264.7 cells. Specifically, the positively charged ZnO NPs exerted higher cytotoxicity than the negatively charged ones. Next, to gauge systemic immunotoxicity, we assessed immune responses of C57BL/6 mice after oral administration of 750 mg/kg/day dose of ZnO NPs for 2 weeks. In parallel, ZnO NPs did not alter the cell-mediated immune response in mice but suppressed innate immunity such as natural killer cell activity. The CD4<sup>+</sup>/CD8<sup>+</sup> ratio, a marker for matured T-cells was slightly reduced, which implies the alteration of immune status induced by ZnO NPs. Accordingly, nitric oxide production from splenocyte culture supernatant in ZnO NP-fed mice was lower than control. Consistently, serum levels of pro/anti-inflammatory (interleukin [IL]-1 $\beta$ , tumor necrosis factor- $\alpha$ , and IL-10) and T helper-1 cytokines (interferon- $\gamma$  and IL-12p70) in ZnO NP-fed mice were significantly suppressed. Collectively, our results indicate that different sized and charged ZnO NPs would cause in vitro and in vivo immunotoxicity, of which nature is an immunosuppression.

**Keywords:** immunosuppression, cytokine, ZnO, immune response, cytotoxicity, innate immunity

## Introduction

Nanotechnology has enabled nanoparticles (NPs) to be designed at the molecular (nanometer) level. Thus, NPs have received the tremendous advantage of their small size, novel physicochemical properties, as well as their interactions with biological systems. In particular, inorganic NPs with metal oxides have been pre-clinically employed for diagnostic and therapeutic use in biomedicine.<sup>1-4</sup> Of these metal oxides, zinc oxide (ZnO) NPs have received considerable attention, with a promising biological application for drug delivery and cancer therapy<sup>5-7</sup> due to their great photo-catalytic and photo-oxidizing ability against chemical and biological species.

Despite the potential biomedical application of ZnO NPs, biohazards and toxicities of ZnO NPs remain unclear. Of these toxicities, the effects of ZnO NPs on the immune system are poorly documented. Here, immunotoxicity is defined as the adverse effects on the immune system such as hypersensitivity, chronic inflammation, immunosuppression, immunostimulation, and autoimmunity. Much evidence suggests that ZnO

NPs would function as immunotoxicants.<sup>8–11</sup> ZnO NPs have unique physicochemical properties, therefore, they could easily access several immune tissues and cells through various routines such as inhalation, ingestion, skin uptake, and injection. ZnO NP oral administration could cause severe damage to heart, lung, liver, and kidney,<sup>12</sup> consequently leading to inflammation. In addition, in vitro exposure of ZnO leads to generation of reactive oxygen species (ROS), upregulation of genes involved in apoptosis, and cell death in human keratinocyte HaCaT cells.<sup>13,14</sup> Likewise, profuse release of inflammatory mediators induced by ZnO NPs<sup>15,16</sup> may result in immune stimulation or aggravation of immune diseases<sup>17</sup> or even break down T helper (Th)-1/Th-2 balance.<sup>18</sup>

It is well known that immunotoxicity of NPs is intimately linked to oxidative stress. For instance, the toxicity of ZnO NPs to immune cells is involved in ROS generation.<sup>19–22</sup> The high production of superoxide in mitochondria reduces the mitochondrial membrane potential,<sup>23</sup> causing cell cycle arrest at S/G2 phase,<sup>24</sup> and increases the ratio of Bax/Bcl-2, leading to the mitochondria-mediated pathway involved in apoptosis.<sup>25</sup> However, the mechanism of immunotoxicity of ZnO NPs in relation to oxidative stress is unclear.

To trigger immunotoxicity, several traits or features of NPs are essential.<sup>26</sup> Emerging evidence implies that the toxicity of ZnO NPs could be affected by size and/or electrostatic charge.<sup>27,28</sup> For instance, an increase of ZnO NPs size might conversely decrease their toxicities.<sup>19,29</sup> Positively charged NPs could exert higher immunotoxicity than negatively charged NPs due to effective interaction with the negative charge of acidic acid on the surface of macrophages.<sup>30</sup> However, these studies<sup>27,28</sup> failed to address whether immunotoxicity of ZnO NPs would be affected by the size and/or charge and whether this immunotoxicity could be categorized as immunostimulation and/or immunosuppression.

In this study, we explored the in vitro potential immunotoxicity of ZnO NPs on Raw 264.7 cells, and the systemic in vivo immunotoxicity of ZnO NPs using C57BL/6 mice. Further, we investigated the role of size and charge in ZnO NP-induced immunotoxicity.

## Material and methods

### Preparation of particle suspensions

ZnO (ZnO-310, Lot No 141319) was purchased from Sumitomo Osaka Cement Co, Ltd, (Tokyo, Japan). ZnO<sup>AE100</sup> (Zn-OX-01-NP.100N, Lot No 1871511079-673) was purchased from American Elements (Los Angeles, CA, USA). The ZnO NPs used in this study were sized at 20 nm (ZnO<sup>SM20</sup>, Sumitomo Osaka Cement Co, Ltd) and 100 nm (ZnO<sup>AE100</sup>;

American Elements), with approximately 99.5% purity, milky white color, and nearly spherical shape. According to the manufacturer information, the actual size and zeta potential of the ZnO NPs used in this study were as follows: 29±3 nm, -44.4±1 mV (ZnO<sup>SM20(-)</sup>); 35±5 nm, 26.3±0.5 mV (ZnO<sup>SM20(+)</sup>); 72±11 nm, -41.6±0.6 mV (ZnO<sup>AE100(-)</sup>); and 79±12 nm, 26.1±0.4 mV (ZnO<sup>AE100(+)</sup>). The surface charge (zeta potential) was modified with coating reagents, citrate to make the ZnO NPs negatively charged and L-serine to make them positively charged, as reported previously.<sup>31</sup> In brief, 20 mg dry powder of ZnO NPs was dissolved into 100 mL of L-serine/HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 6.2, and citrate/HEPES pH 7.3 to make the NP surfaces electrostatically charged. Indicated buffers were made as follows: for the positive charge buffer, 99 mL of 20 mM HEPES pH 6, L-serine (1 g) adjusted to pH 6.2, and for the negative charge buffer, 99 mL of 20 mM HEPES pH 7, sodium citrate (1 g) adjusted to pH 7.3. Subsequently, the ZnO NP suspension was vortexed for 5 minutes at room temperature and then kept at 4°C until use. Before using, the suspension was sonicated at 4°C for 10 minutes with a sonicator (Hielscher-Ultrasound Technology, Teltow, Germany).

### Cell culture

Raw 264.7, mouse macrophage cell line (American Type Culture Collection [ATCC], Manassas, VA, USA) was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone Laboratories, Inc., South Logan, UT, USA) supplemented with 10% heat-activated fetal bovine serum (FBS, Hyclone Laboratories Inc.) and 1% antibiotic-antimycotic (Gibco®, Invitrogen Corporation, Carlsbad, CA, USA) at 37°C in a 5% CO<sub>2</sub> incubator.

### In vitro cell viability study

A commercially available cell viability assay Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was employed to evaluate the cytotoxic effect of ZnO NPs. Approximately 1×10<sup>5</sup> of Raw 264.7 cells was seeded into 96-well plates then incubated with various concentrations of ZnO NPs for 24 hours at 37°C in a 5% CO<sub>2</sub> incubator. Cells not treated with ZnO NPs served as a control well in the experiment. Non-modified zinc chloride (ZnCl<sub>2</sub>) dissolved in 1X phosphate buffered saline (PBS) was used as a control, without modification of surface. Afterwards, 10 µL of CCK-8 solution was added to each well, incubated for 1 hour, and then absorbance was determined at 450 nm by a DTX-880 multimode microplate reader (Beckman Coulter, Fullerton,

CA, USA). The percentage of cell viability was calculated by the following formula: cell viability (%) = (mean absorbency in test wells)/(mean absorbency in control wells) × 100. All experiments were performed in triplicate.

## Maintenance of animals and ZnO NP treatment

Six-week-old inbred C57BL/6 mice (n=5) were purchased from Orient Bio Inc. (Seongnam, South Korea) and were maintained in a pathogen-free condition, and fed with a standard commercial diet. Mice were randomly assigned into five groups: normal control group, which was PBS treated, and four experimental groups treated with four types of ZnO NPs. Briefly, the ZnO NP suspension was orally administered in the mice with the dosage of 750 mg/kg every day continuously for 14 days. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Wonju College of Medicine, Yonsei University.

## Induction and evaluation of delayed-type hypersensitivity (DTH)

Mice were orally administered with 750 mg/kg of different sized and charged ZnO NPs for 2 weeks. DTH assays were performed 4 days before sacrifice. To assess DTH response, mice were subcutaneously injected in the left footpad with 20  $\mu$ L of saline as a control, and in the right footpad with 20  $\mu$ L of ZnO NP suspension. At the indicated times after challenge (24 hours and 48 hours), footpad thickness was measured with a digital caliper (Mitutoyo Corporation, Tokyo, Japan). The level of the DTH response was determined as the difference between the left and right footpad.

## Preparation of splenocytes

To isolate splenocytes, the spleen was removed aseptically from C57BL/6 mice at the endpoint treatment and was placed in a tube containing 1× PBS on ice. Splenocyte suspensions were prepared by gently pressing the spleen between the frosted ends of two sterile microscope slides into a 100 mm tissue culture grade petri dish. The slides were rinsed at regular intervals with 1× PBS. Cell suspensions were filtered by a sterile plastic strainer and then centrifuged at 1,500 rpm for 3 minutes. For optimal lysis of erythrocytes, the pellets were resuspended in 5 mL 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, the 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 1X PBS and resuspended

in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 3% FBS (Hyclone Laboratories Inc.) and 1% antibiotic-antimycotic (Invitrogen Corporation). 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).

## Splenocyte proliferative responses to concanavalin A (Con A) and lipopolysaccharide (LPS)

Splenocytes were seeded at  $1 \times 10^5$  cells per well into a 96-well, flat-bottom microtiter plate in 100  $\mu$ L RPMI-1640 (Hyclone Laboratories, Inc.) supplemented with 10% heat-activated FBS (Hyclone Laboratories Inc.) and 1% antibiotic/antimycotics. Thereafter, 1.25  $\mu$ g/mL Con A (Sigma-Aldrich, St Louis, MO, USA) and 500  $\mu$ g/mL of LPS (Sigma-Aldrich) were used. The plates were incubated at 37°C in a humidified atmosphere under 5% CO<sub>2</sub> for 6 hours. Cell proliferation was evaluated using a CCK-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Absorbance was measured at 450 nm by a DTX-880 multimode microplate reader (Bechman Counter Inc.).

## Cytotoxicity assay of natural killer (NK)-cells

NK-enriched murine splenocytes were used as effector cells, and YAC-1 (ATCC) as target cells. Briefly, various effector cell dilutions were prepared:  $1 \times 10^6$  cells/well for 100:1,  $5 \times 10^5$  cells/well for 50:1, and  $2.5 \times 10^5$  cells/well for 25:1. A constant number of target cells ( $1 \times 10^3$  cells/well) were then co-cultured with the different effector cell dilutions prepared in a 96-well plate to test several effector/target cell ratios. The final combined volume was 100  $\mu$ L/well. The effector/target cells were incubated 6 hours at 37°C humidified incubator with 5% CO<sub>2</sub>. The cytotoxic activity of NK-cells was assessed by a Cytotox 96 non-radioactive cytotoxicity assay (Promega Corporation, Fitchburg, WI, USA). Briefly, 50  $\mu$ L supernatant from the cell culture was collected and was moved to the new 96-well round-bottom plate. Then, 50  $\mu$ L of the lactate dehydrogenase (LDH) substrate mixture was added to each well and incubated for 30 minutes at room temperature, protected from light. The LDH that was released upon cell lysis was measured in the supernatant by optical density (OD) measurement at 490 nm. Target cell lysis was calculated as: (OD of sample – OD with spontaneous release of LDH from target cells – OD with spontaneous release of LDH from effector cells) × 100/(OD with maximal release of LDH from target cells – OD with spontaneous release of

LDH from target cells). All experiments were performed in triplicate.

## Immunophenotyping of splenocytes

Specific leukocyte subtypes of cells derived from mouse spleen were also determined by immunofluorescent antibody staining and analyzed with flow cytometry. Lymphocyte subpopulations were identified and gated using forward versus side scatter characteristics. All monoclonals were directly conjugated and were obtained from BD Biosciences (San Jose, CA, USA). Th cells (CD4+), cytotoxic T-cells (CD8+), T-cells (PE-CD4+, FITC-CD8+), B-cells (PE-CD19+, FITC-B220[CD45R+]), NK-cells (CD16+), macrophages (PE-CD11b+; FITC-CD14+), and monocytes (CD14+) were identified using the anti-mouse antibodies. Thereafter, approximately  $5 \times 10^5$  cells were resuspended in flow cytometry buffer (2% FBS, 0.02% sodium azide in PBS) containing Fc-block to reduce nonspecific antibody binding. Cells were then incubated in the dark with the appropriate fluorochrome-conjugated antibody (10  $\mu$ L of 1  $\mu$ g/mL) for 30 minutes at 4°C. Afterwards, cells were washed twice with 500  $\mu$ L FACS (fluorescence activated cell sorter) buffer, and flow cytometry analysis was performed on the Cytomics™ FC 500 Flow Cytometer (Beckman Coulter). Control samples were matched for each fluorochrome. Data were analyzed using Modfit LT 3.2 (Verity Software House, Topsham, ME, USA) software.

## Measurement of nitric oxide (NO)

NO production in the primary splenocyte culture medium was quantified spectrophotometrically using the Griess reagent G2930 (Promega Corporation). The nitrite ( $\text{NO}_2^-$ ) present in the supernatant of splenocytes was used as an indicator of NO. Nitrite is a stable degradation product of NO. Briefly, 50  $\mu$ L of the splenocyte supernatant was mixed with an equal volume of Griess reagent in a 96-well round-bottom microtiter plate and incubated at room temperature for 15 minutes. The absorbance at 540 nm was measured, and the NO concentration was determined using a calibration curve, with sodium nitrite as a standard chemical.

## Measurement of serum cytokine level

The level of the cytokines (interleukin [IL]-1 $\beta$ , IL-6, tumor necrosis factor [TNF]- $\alpha$ , interferon [IFN]- $\gamma$ , IL-12p70, and IL-10) in serum was determined using Multiplex Bead Suspension Array System (Bio-Rad Laboratories, Hercules, CA, USA). The cytokine multiplex bead suspension array kit was purchased from Bio-Rad Laboratories. Briefly, each

set of premixed beads coated with the target antibodies was added to a well, and 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 specific incubation time, with shaking at room temperature and washing steps. All washes were performed using a Bio-Plex Pro™ (Bio-Rad Laboratories) wash station. Finally, the samples were then read using the Bio-Plex suspension array reader, and data acquisition and analysis were carried out with the five-parameter logistic method.

## Statistical analysis

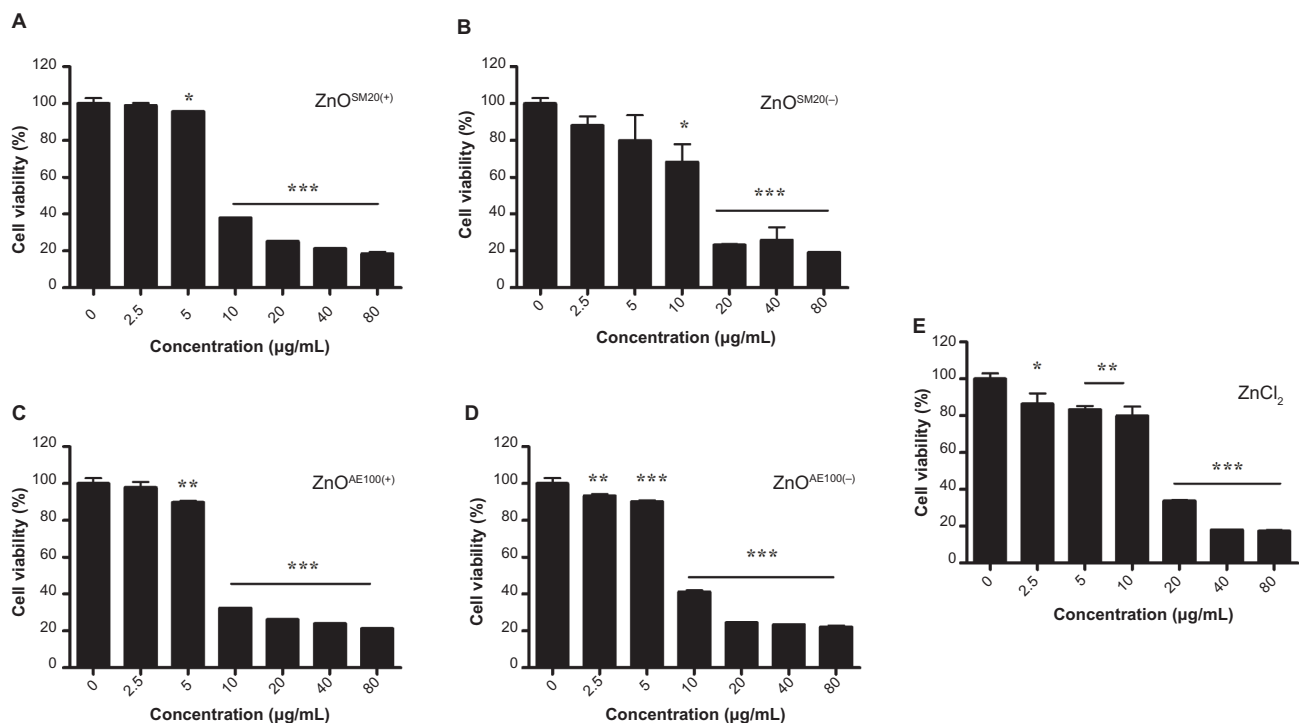
All data are presented as the mean  $\pm$  standard error of the mean. The mean values among different groups were analyzed and compared using one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey) with Graph Prism (GraphPad Software, La Jolla, CA, USA) version 5.0 software packages. One-way ANOVA with repeated measurements followed by Tukey's test was applied to test the influence of ZnO NPs on body-weight gain. A  $P \leq 0.05$  was considered to be statistically significant.

## Results

### Cell viability study

The influence of ZnO NP size and charge on immune cells remains unclear. To clarify this, using four types of ZnO NPs, we examined in vitro cell viability. The effect of ZnO NPs on the viability of Raw 264.7 cells was examined using CCK-8 assay, and  $\text{ZnCl}_2$  was used as a positive control group (Figure 1). ZnO NPs at the concentrations 0–5  $\mu$ g/mL had minimal effect on the viability of Raw 264.7 cells, although they significantly reduced the viability at a higher concentration range (10–80  $\mu$ g/mL) after 24 hours incubation. To compare the potency of each ZnO NP, the half maximal effective concentration ( $\text{EC}_{50}$ ) value was calculated according to sigmoidal dose–response regression (Table 1). The range of  $\text{EC}_{50}$  of ZnO NPs was 6.584–8.413  $\mu$ g/mL:  $\text{ZnO}^{\text{AE100}(+)}$  exerted the highest cytotoxicity against Raw 264.7 cells ( $\text{EC}_{50} = 7.499$   $\mu$ g/mL), while the cytotoxicity strength of other ZnO NPs was  $\text{ZnO}^{\text{AE100}(-)} > \text{ZnO}^{\text{SM20}(+)} > \text{ZnO}^{\text{SM20}(-)} > \text{ZnCl}_2$  in descending order. Of note,  $\text{ZnCl}_2$  was less cytotoxic than ZnO NPs ( $\text{EC}_{50} = 14.82$   $\mu$ g/mL). Additionally,  $\text{EC}_{50}$  values of 100 nm-sized ZnO NPs were higher than those of





**Figure 1** Effect of different sized and electrostatically charged ZnO NPs on the viability of Raw 264.7 cells. Cells were incubated, with indicated concentrations of ZnO NPs for 24 hours: (A) ZnO<sup>SM20(+)</sup>, (B) ZnO<sup>SM20(-)</sup>, (C) ZnO<sup>AE100(+)</sup>, (D) ZnO<sup>AE100(-)</sup>, and (E) ZnCl<sub>2</sub>. DMEM media was used as a negative control. Cell viability was then determined by a CCK-8 assay.

**Notes:** All values are presented as mean  $\pm$  SEM of three experiments conducted in triplicate. Percentages were also calculated in reference to the control well (0). \* $P < 0.05$ ; \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  versus control well (0). AE = American Elements (Los Angeles, CA, USA); CCK-8 = Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA); SM = Sumitomo Osaka Cement Co, Ltd. (Tokyo, Japan).

**Abbreviations:** DMEM, Dulbecco's Modified Eagle's Medium; NP, nanoparticle; SEM, standard error of the mean; ZnCl<sub>2</sub>, zinc chloride; ZnO, zinc oxide.

20 nm-sized ZnO NPs. Moreover, positively charged NPs showed higher cytotoxicity compared with their positively charged counterpart, suggesting that the size and charge of ZnO NPs could affect their cytotoxicity.

## Immunophenotyping

ZnO NP treatment slightly induced the alteration in cell distribution of splenocytes (Table 2). While Th (CD4+) and cytotoxic T (CD8+) cells accounted for 16.6% and 9.1%, respectively, of the control, the distribution of Th cells and cytotoxic T-cells in splenocytes of treated groups (ZnO<sup>SM20(+/-)</sup>, ZnO<sup>AE100(+/-)</sup>) was changed to 14.66%/9.18%, 15.44%/11.62%, 14.53%/10.22%, 16.80%/11.60%, respectively. Notably, the percentage of Th cells was signifi-

cantly reduced when treated with ZnO<sup>AE100(+)</sup> as compared with control (Table 2). Moreover, the ratio of CD4+ T-cells to CD8+ T-cells, which are subpopulations of T-cells (Th cells and cytotoxic T-cells), significantly changed from 1.634-fold to 1.301-fold in ZnO<sup>AE100(+)</sup>-fed mice. However, little differences were noted in the proportion of B-cell, NK-cell, macrophage, and monocyte subpopulations.

## Innate, cell-mediated immune response (DTH and mitogenic response) against ZnO NPs

To assess the effect of ZnO NPs on regulation of innate immune response, NK-cell activity was examined using NK-sensitive YAC-1 target cells. As shown in Figure 2,

**Table 1** EC<sub>50</sub> values (24 hours growth inhibition) of ZnO NPs on Raw 264.7 cells

|                              | ZnO         |              |             |             | ZnCl <sub>2</sub> |
|------------------------------|-------------|--------------|-------------|-------------|-------------------|
|                              | SM20(+)     | SM20(-)      | AE100(+)    | AE100(-)    |                   |
| EC <sub>50</sub> (µg/mL)     | 8.419       | 10.710       | 7.499       | 8.166       | 14.820            |
| 95% confidence limit (µg/mL) | 7.109–9.730 | 5.087–16.330 | 6.584–8.413 | 7.124–9.207 | 8.866–20.780      |

**Notes:** All values are presented as mean  $\pm$  SEM of the three experiments conducted in triplicate; (+), positive charge; (-), negative charge. AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co, Ltd. (Tokyo, Japan).

**Abbreviations:** EC<sub>50</sub>, half maximal effective concentration; NP, nanoparticle; SEM, standard error of the mean; ZnCl<sub>2</sub>, zinc chloride; ZnO, zinc oxide.

**Table 2** Immunophenotype of splenocytes in C57BL/6 mice fed with ZnO NPs for 14 days

| Administration                                      | PBS        | ZnO <sup>SM20(+)</sup> | ZnO <sup>SM20(-)</sup> | ZnO <sup>AE100(+)</sup> | ZnO <sup>AE100(-)</sup> |
|-----------------------------------------------------|------------|------------------------|------------------------|-------------------------|-------------------------|
| CD4 <sup>+</sup> CD8 <sup>-</sup> <sup>a</sup>      | 16.60±0.60 | 14.66±0.80             | 15.44±0.54             | 14.53±1.37*             | 16.80±0.69              |
| CD4 <sup>+</sup> CD8 <sup>+</sup> <sup>b</sup>      | 9.10±1.18  | 9.18±0.42              | 11.62±0.53             | 10.22±0.64              | 11.60±0.33              |
| CD4 <sup>+</sup> CD8 <sup>+</sup> <sup>c</sup>      | 0.40±0.04  | 0.32±0.04              | 0.32±0.04              | 0.38±0.11               | 0.28±0.05               |
| B220 <sup>+</sup> CD19 <sup>-</sup>                 | 0.78±0.09  | 0.68±0.04              | 0.80±0.07              | 0.83±0.09               | 0.60±0.03               |
| B220 <sup>+</sup> CD19 <sup>+</sup>                 | 8.70±1.09  | 7.82±0.38              | 7.20±0.42              | 9.02±1.32               | 5.98±2.23               |
| B220 <sup>+</sup> CD19 <sup>+</sup> <sup>d</sup>    | 51.58±1.56 | 51.78±2.52             | 53.70±0.54             | 53.50±0.55              | 52.92±1.04              |
| CD16 <sup>+</sup> <sup>e</sup>                      | 58.55±1.30 | 59.02±2.26             | 59.88±0.53             | 55.44±6.69              | 58.76±1.17              |
| CD11b <sup>+</sup> CD14 <sup>-</sup>                | 6.80±0.27  | 6.54±0.22              | 6.80±0.34              | 6.52±0.71               | 5.56±0.23               |
| CD11b <sup>-</sup> CD14 <sup>+</sup>                | 0.25±0.03  | 0.42±0.06              | 0.26±0.06              | 0.30±0.03               | 0.30±0.04               |
| CD11b <sup>+</sup> CD14 <sup>+</sup> <sup>f,g</sup> | 1.23±0.06  | 1.04±0.07              | 1.04±0.12              | 1.50±0.21               | 0.94±0.07               |

**Notes:** Values are presented as mean ± SEM, n=5; <sup>a</sup>T helper cells; <sup>b</sup>Cytotoxic T-cells; <sup>c</sup>T-cells; <sup>d</sup>B-cells; <sup>e</sup>NK-cells; <sup>f</sup>Macrophages; <sup>g</sup>Monocytes; \*Nonparametric Tukey's multiple comparison test,  $P < 0.05$  versus PBS. AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co, Ltd, (Tokyo, Japan).

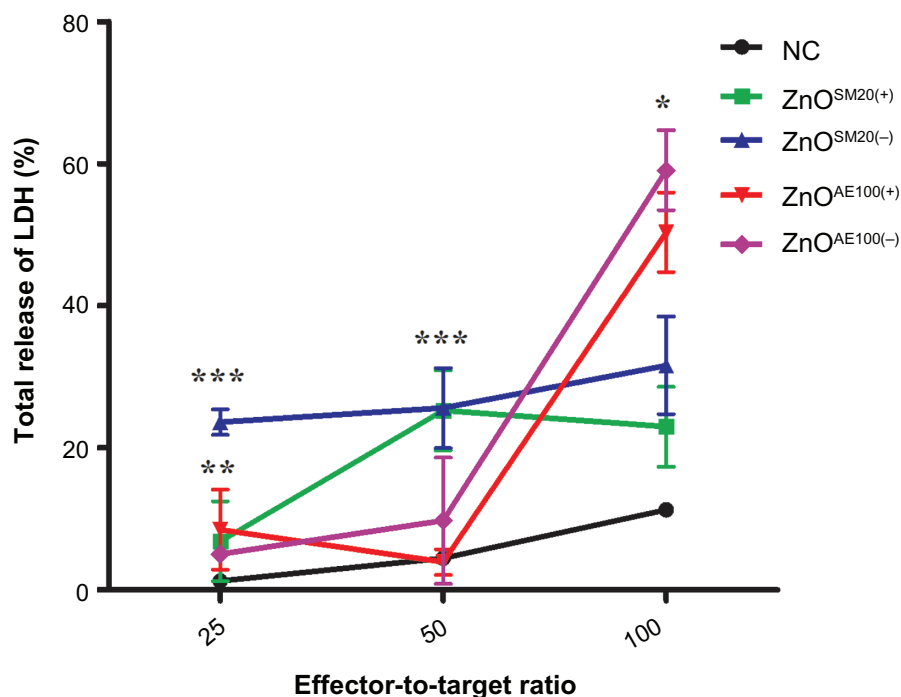
**Abbreviations:** NP, nanoparticle; PBS, phosphate buffered saline; SEM, standard error of the mean; ZnO, zinc oxide.

a decrease of NK-cell activity was observed in the mice treated with ZnO NPs. At the ratio of 100:1, ZnO NPs significantly inhibited NK-cell activity. Next, we examined the effect of ZnO NPs on cell-mediated immunity (CMI) using DTH response on C57BL/6 mice. The swelling volume was similar after 24-hour and 48-hour challenges (Table 3). An increased DTH response to ZnO NPs was observed. However these differences were not statistically significant. Further, we analyzed the mitogen-stimulated proliferative responses

of T- and B-lymphocytes (Figure 3). These responses were elevated compared with control but not significant. Taken together, ZnO NPs affected NK activity but not CMI.

## NO production of splenocytes

NO is a reactive form of nitrogen and is an important cellular signaling molecule involved in several biological processes, and moreover, serves as one of the key mediators of immune defense. To further explore the impact of ZnO NPs



**Figure 2** Effects of ZnO NP treatment on NK-cell activity. Effector cells (NK-cells) were isolated from the spleen of mice fed with a 750 mg/kg dose of ZnO NPs for 14 days. YAC-1 cells were used as target cells. ZnO NP treatment reduced NK-cell cytotoxicity in the treated groups compared with control.

**Notes:** Values are presented as mean ± SEM of percentage cytotoxicity, n=5. \* $P < 0.05$ ; \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co, Ltd, (Tokyo, Japan).

**Abbreviations:** LDH, lactate dehydrogenase; NC, normal control; NK, natural killer; NP, nanoparticle; SEM, standard error of the mean; ZnO, zinc oxide.

**Table 3** Delayed type hypersensitivity in ZnO NP-primed mice

| Treatment               | Foot-pad swelling (mm) |              |               |               |
|-------------------------|------------------------|--------------|---------------|---------------|
|                         | 24 hours               |              | 48 hours      |               |
|                         | Saline                 | ZnO NPs      | Saline        | ZnO NPs       |
| ZnO <sup>SM20(+)</sup>  | 6.780±1.484            | 7.242±1.318  | 6.8230±0.3485 | 7.8120±0.7510 |
| ZnO <sup>SM20(-)</sup>  | 1.5230±0.9862          | 1.202±1.103  | 1.5570±0.3313 | 1.7060±0.5497 |
| ZnO <sup>AE100(+)</sup> | 10.5500±0.4293         | 11.700±2.053 | 7.1400±0.6409 | 10.480±1.521  |
| ZnO <sup>AE100(-)</sup> | 4.3170±0.8631          | 4.718±1.730  | 1.3400±0.7702 | 1.3380±0.6657 |

**Notes:** Measurement of footpad swelling in mice induced by DTH response after 24-hour and 48-hour challenges. Values are presented as mean ± SEM, n=5. AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co. Ltd, (Tokyo, Japan).

**Abbreviations:** DTH, delayed-type hypersensitivity; NP, nanoparticle; SEM, standard error of the mean; ZnO, zinc oxide.

on immune defense, we measured the level of splenic NO production after 14 days treatment. As shown in Figure 4, a significant decrease in NO level occurred after administration of ZnO<sup>SM20(-)</sup> and ZnO<sup>AE100(+/-)</sup>. However, there was no substantial difference of NO level in ZnO<sup>SM20(+)</sup>-fed mice as compared with the control.

## Serum cytokine level

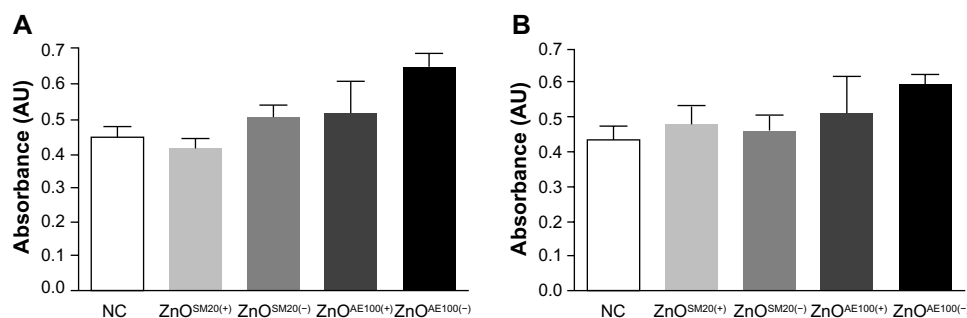
To explore immune status change induced by ZnO NPs we examined serum pro- and anti-inflammatory cytokine (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-12p70, IFN- $\gamma$ , and IL-10) levels at the end-point treatment. We found that overall, the level of serum cytokines in ZnO-fed mice was lower than saline-fed mice (Figure 5). In particular, serum levels of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , and IL-12p70) in ZnO NP-fed mice were ZnO<sup>SM20(+)</sup> > ZnO<sup>SM20(-)</sup> > ZnO<sup>AE100(+)</sup> > ZnO<sup>AE100(-)</sup> in descending order. Accordingly, the level of IL-10, an anti-inflammatory cytokine in ZnO NP-fed mice tended to be significantly lower than PBS-fed mice. By contrast, there was no significant change of cytokine level in ZnO<sup>SM20(+)</sup>-fed mice. In terms of size and charge effect on serum cytokine level, the size and charge were correlated to serum cytokine level. Further, the size appears more correlated to the serum

cytokine than the charge. Taken together, our data show that oral intake of ZnO NPs in mice could drop the level of serum cytokines, importantly implying the possible suppression of immune status of C57BL/6 mice fed with ZnO NPs.

## Discussion

Convincing evidence has shown that ZnO NPs would have a potential to be accumulated to several organs such as liver, spleen, kidneys, brain or heart after deliberately placed in the body.<sup>32</sup> In this regard, ZnO NPs would encounter diverse immune cells in the human body, thus damaging immune tissues and organs. However, the immunotoxicity of ZnO NPs in relation to the size and charge of ZnO NPs is unclear. To address this issue, we assessed the immunotoxicity of ZnO NPs in vitro and in vivo and explored their underlying mechanism. First, the in vitro study indicates that ZnO NPs induce cytotoxicity to immunocytes via cell viability assay, which is influenced by the size and charge of ZnO NPs. To validate this, we examined cell viability using colorimetric (CCK-8 assay) in Raw 264.7, macrophage cell line.

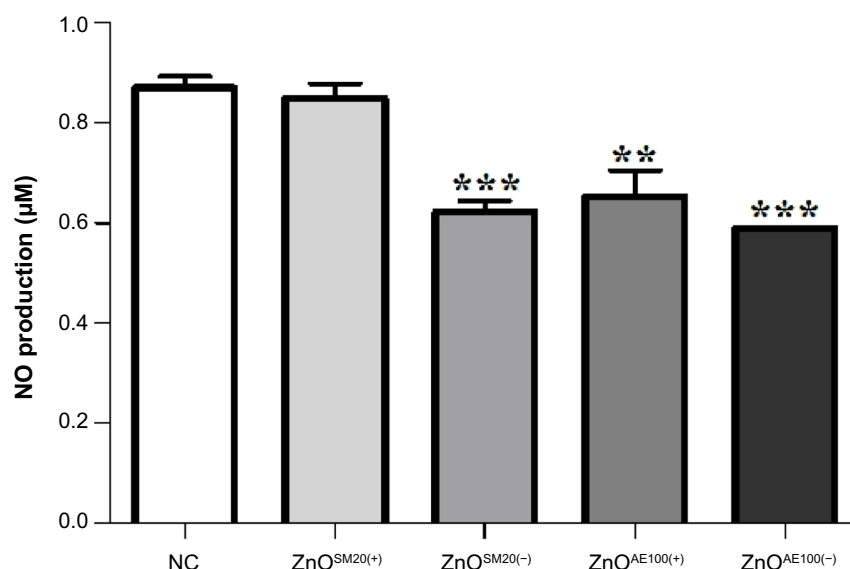
Cell viability assay showed that ZnO NPs with different size and electrostatic charge have differential cytotoxicity to Raw 264.7 cells (Figure 1). Interestingly, the positively



**Figure 3** Mitogenic response of mouse splenocytes. Splenocytes ( $1 \times 10^6$  cells/mL) were incubated for 24 hours with concanavalin A or lipopolysaccharide to evaluate T-lymphocyte (A) and B-lymphocyte (B) proliferation, respectively.

**Notes:** Values are presented as mean ± SEM, n=5. AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co. Ltd, (Tokyo, Japan).

**Abbreviations:** NC, normal control; SEM, standard error of the mean; ZnO, zinc oxide.



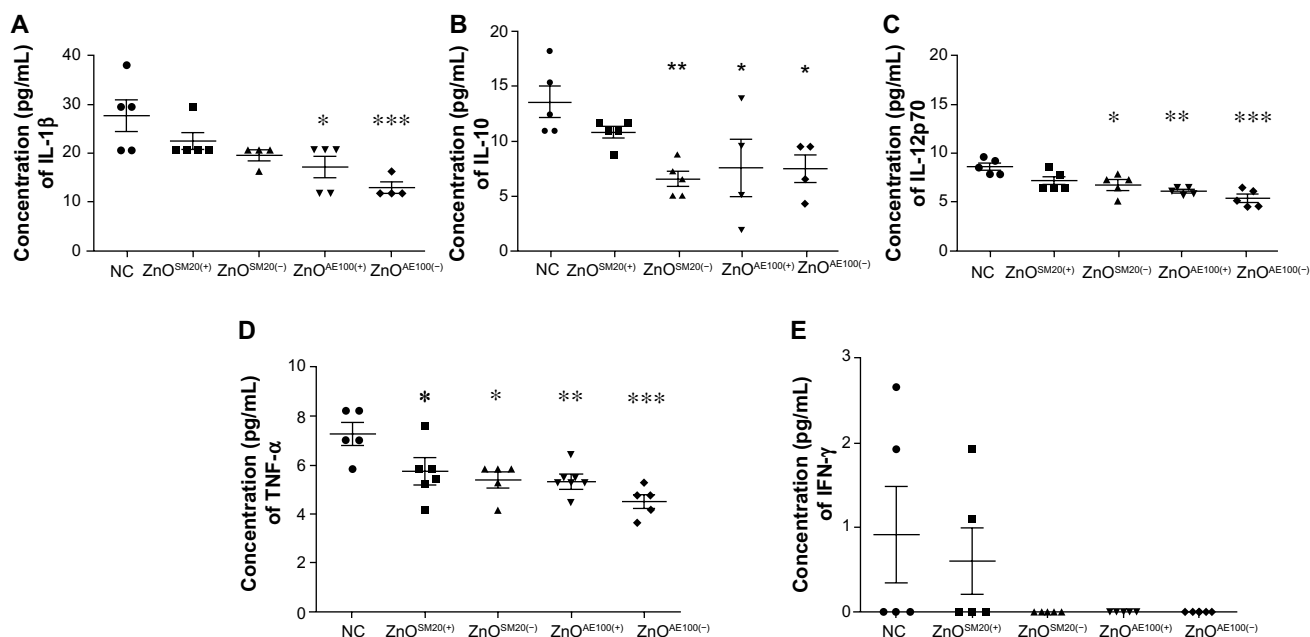
**Figure 4** Detection of NO induced by ZnO NPs on mouse splenocytes. Splenocytes were isolated and cultured. NO production in the supernatant was measured by the Griess reaction.

**Notes:** Values are presented as mean  $\pm$  SEM,  $n=5$ . \*\* $P<0.01$ , and \*\*\* $P<0.001$ . AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co, Ltd, (Tokyo, Japan).

**Abbreviations:** NC, normal control; NO, nitric oxide; NP, nanoparticle; SEM, standard error of the mean; ZnO, zinc oxide.

charged NPs exerted higher cytotoxicity against Raw 264.7 cells than the negatively charged ones. ZnO NP-induced cytotoxicity could be affected by the release of  $Zn^{2+}$  ions through the dissolution of ZnO NPs within aqueous culture media.<sup>33–35</sup> In terms of  $EC_{50}$ , we found that ZnO NPs would

cause cytotoxicity at lower doses (Table 1). This difference might be due to inherent features of metal oxides: free  $Zn^{2+}$  ions and oxidative radical-generated oxide, and ZnO NP surface charge, would synergistically exert cytotoxicity to eukaryotic cells. In this experiment, another merit is the



**Figure 5** ZnO NPs suppress cytokine release. After 14 days exposure, mice were retro-orbital bled, and serum was isolated by centrifugation and examined for levels of IL-1 $\beta$  (A), IL-10 (B), IL-12p70 (C), TNF- $\alpha$  (D), and IFN- $\gamma$  (E) using Multiplex Bead Array System (Bio-Rad Laboratories, Hercules, CA, USA).

**Notes:** Values are presented as mean  $\pm$  SEM,  $n=5$ . \* $P<0.05$ ; \*\* $P<0.01$ , and \*\*\* $P<0.001$ . AE = American Elements (Los Angeles, CA, USA); SM = Sumitomo Osaka Cement Co, Ltd, (Tokyo, Japan).

**Abbreviations:** IFN, interferon; IL, interleukin; NC, normal control; NP, nanoparticle; SEM, standard error of the mean; TNF, tumor necrosis factor; ZnO, zinc oxide.



validity of in vitro toxicity test using the Raw 264.7 cell line. While, this cell line is a well-known standard cell line for in vitro toxicity testing, in vitro toxicity testing for nanoparticles has not been established. Collectively, we have demonstrated that macrophage Raw 264.7 cells exposed to ZnO NPs show different sized- and charged-dependent cytotoxicity, as was observed by others.<sup>19,36,37</sup> Further, the dose-dependent decrease in cell viability by exposure with ZnO NPs might be related to the dissolution and release of  $Zn^{2+}$  ions, as previously observed in in-vitro studies.<sup>33–35,38–40</sup> This  $Zn^{2+}$  ion would affect cellular differentiation and cellular metabolism solely or via the interaction with proteins in vitro and in vivo.

Since, in vitro culture cannot reflect the complexity of an in vivo system, which is preferred for the toxicological evaluation, we next examined the immunotoxicological parameters induced by oral intake of ZnO NPs in mice. Our in vivo study indicates that the different sized- and charged-ZnO NPs could cause immunotoxicity in ZnO NP-fed mice, of which nature is an immunosuppression. This stems from our immunotoxicological data. Prior to immunotoxicological evaluation, we checked the weight-related parameters (bodyweight and coefficient of spleen to bodyweight). Of these, only the reduced bodyweight gain was noted (data not shown), suggesting the potential immunotoxicity of ZnO NPs in vivo. Since in vitro data showed ZnO-induced toxicity in immunocytes, bodyweight change coupled with in vitro data might be considered for predicting potential immunotoxicity of nanoparticles.

To explore the effect of ZnO NPs on the CMI, we measured the DTH reaction to ZnO NPs, splenocyte proliferation in response to Con A and LPS, NK-cell activity, and splenocyte phenotyping. Overall, no significant change was observed in DTH responses (Table 3) and T- and B-cell proliferation (Figure 3). By contrast, we found a slight change in splenocyte phenotypes (Table 2). Since CD4+ cells can help the proliferation and differentiation of Th cells, and CD8+ cells can exert cytotoxicity and regulate CD4+ cells, the ratio of CD4+/CD8+ can reflect the overall immune status in the host. Of note, there was the decreased percentage of CD4+ Th cell subpopulation in  $ZnO^{AE100(+)}$ -fed mice as compared with control mice (Table 2). Consistently, the ratio of CD4+/CD8+ was reduced, which might imply systemic immune suppression. These CMI variables do not reveal specific function of the innate arm in CMI. Moreover, CD16 (FcγRIII) alone can be a marker for NK, as this surface marker is shed from the surface after activation of NK-cells.<sup>41,42</sup> Only  $ZnO^{AE100(+)}$ -fed mice showed a decreased

level of CD16 among other groups fed with ZnO. Collective evidence showed that loss of CD16 on NK-cells is associated with reduced antibody-mediated cellular cytotoxicity and weaker antitumor response.<sup>43</sup> Therefore, levels of CD16 could influence NK-cell function. To address this, we checked NK-cell activity. Astonishingly, NK-cell activity at the ratio of 100:1 was significantly decreased in all ZnO NP-fed mice compared with control (Figure 2) and most prominent on the  $ZnO^{AE100(+)}$ -fed group. However, further research is needed to prove this inference, such as development of tumors. NK-cells were defined as lymphocytes mediating cytotoxicity against certain tumors and virus-infected target cells. In this point, the suppression of NK-cell activity may reveal the weakened innate defense in CMI, consequently getting vulnerable to opportunistic dangers such as infection, cancer, and stress. Given this, we hypothesized that if these data would be valid, humoral immune mediators should be co-regulated toward the immune suppression. To verify this, we selected two humoral immune parameters (NO and cytokines).

First, we checked NO production from splenocytes of ZnO-fed mice. NO is a reactive nitrogen species which plays an important role in the destruction and suppression of many intracellular pathogenic organisms.<sup>44,45</sup> NO acts as an effector molecule in macrophage-mediated cytotoxicity.<sup>46</sup> However, the NO production of macrophages was regulated and required to be optimized by CD4+ T-cells. We found that three oral intakes of ZnO NPs, except  $ZnO^{SM20(+)}$ , significantly decreased NO production of splenocytes (Figure 4). Viewed together, this is in line with the suppression of NK-cell activity and the ratio of CD4+/CD8+ observed.

Since NO could contribute to the regulation of immune reaction by modifying the release of cytokines,<sup>47–49</sup> finally we quantified the serum cytokine release in ZnO-fed mice. To further analyze the several facets of in vivo immunotoxicity in ZnO NP-fed mice, we selected five kinds of pro- and anti-inflammatory cytokines (IL-1β, TNF-α, IL-12p70, IFN-γ, and IL-10). Cytokine assay showed that the overall level of serum cytokines in ZnO-fed mice decreased as compared with control (Figure 5). Of these cytokines, in line with the concentration in serum, IL-1β and IL-10 were prominent in detecting the cytokine change in ZnO NP-fed mice. These cytokines were the representative pro- and anti-inflammatory cytokines, respectively. The decreased level of these opposite cytokines in ZnO NP-fed mice might be ascribed to nonspecific immune suppression or even an imbalance between pro- and anti-inflammatory cytokine networks. The limitation of this study is the lack of different dose usage

in vivo. To gauge this phenomena in detail, further work is underway.

## Conclusion

Synthesizing these immunotoxicological data in vivo and in vitro, our results indicate that different sized and charged ZnO NPs would cause in vitro and in vivo immunotoxicity, of which nature is a minor immunosuppression. This has important implications for individuals who may be chronically exposed to ZnO NPs. Further, this study might offer the possibility of the new immune parameters such as cytokine and NO for gauging immunotoxicity for nanoparticles.

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## Disclosure

The authors report no conflicts of interest in this work.

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