



Letter

Potency shift in immunomodulatory activities of zinc oxide (ZnO) nanoparticles in THP-1 cells is associated with cytotoxicity

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ABSTRACT — Two zinc oxide nanoparticles (ZnO NPs) with different physicochemical properties (ZnO(α) and ZnO(Σ)) were examined in THP-1 cells to investigate their effects on cellular immunomodulation and cytotoxicity. THP-1 cells were cultured in the presence of ZnO(α) or ZnO(Σ) for 48 hr, and the expression of proinflammatory cytokines and immune cell surface antigens was examined. ZnO(α) and ZnO(Σ) reduced cell viability in a concentration- and time-dependent manner, with the latter being more potent. ZnO(α) and ZnO(Σ) increased the expression of CD54, IL-8, and TNF- α to the same extent between 24 and 48 hr. While ZnO(Σ) was more potent at effective concentrations, this potency was comparable between ZnO(α) and ZnO(Σ) when normalized to their cytotoxic concentrations (LC₅₀, LC₂₅, or LC₅). It was considered that there was a potency shift that is associated with cytotoxicity and physicochemical properties, in immunomodulatory activities in THP-1 cells between ZnO NPs.

Key words: Zinc oxide, THP-1, Nanoparticle, Cytotoxicity, Immunomodulatory activity

INTRODUCTION

Zinc oxide nanoparticles (ZnO NPs) are widely used in industrial applications, including cosmetics, pharmaceuticals, electronics, and biomedicine, owing to their novel properties compared to bulk ZnO particles (Jiang *et al.*, 2018). While their effects on health and immunity are not fully understood, multiple risk assessments have been conducted (Singh, 2019; Kim *et al.*, 2017).

ZnO NPs have been shown to influence immunomodulatory activities both *in vivo* (Roy *et al.*, 2013; Ha *et*

al., 2016; Jatana *et al.*, 2017) and *in vitro* (Hanley *et al.*, 2009; Feltis *et al.*, 2012; Saptarshi *et al.*, 2015; Sahu *et al.*, 2014; Senapati *et al.*, 2015) studies. However, ZnO NPs have been shown to elicit both adverse (i.e. allergic side effects on skin (Vujovic and Kostic, 2019)) and beneficial (i.e. immune regulation in therapeutic applications (Dukhinova *et al.*, 2019)) reactions, depending on the biomedical application. To advance biomedical applications of ZnO NPs, their immunomodulatory activities must be controlled, possibly through regulation of their physicochemical properties.

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It has been shown that the potency of ZnO NPs in regulating immunomodulatory activities depends on their physicochemical properties. For example, smaller ZnO NPs have been shown to induce expression of proinflammatory cytokines, as a measure of immunomodulatory activity, more potently than larger particles in monocytic THP-1 cells, suggesting the importance of the large surface area due to the nano size (Feltis *et al.*, 2012; Sahu *et al.*, 2014).

A similar relationship was observed by measuring the zeta potential, an index of the surface charge, which had an inverse relationship with the secondary particle size, an index of the aggregation state, suggesting the involvement of phagocytic efficiency. Therefore, a ZnO NP with a smaller zeta potential or a larger secondary particle size has greater potency (Sahu *et al.*, 2014). Additionally, the cytotoxicity of ZnO NPs depends on their physicochemical properties; however, its relationship with their immunomodulatory activities has yet to be shown (Feltis *et al.*, 2012; Sahu *et al.*, 2014).

In this study, we investigated the effects of two ZnO NPs with different physicochemical properties on immunomodulatory activity and cytotoxicity in human monocytic leukemia THP-1 cells. Specifically, the expression of immune cell surface antigens, CD54 and 86, and proinflammatory cytokines, including TNF- α , were examined. These immune cell surface antigens are known to be involved in the activation of dendritic cells (Aiba *et al.*, 1997), and THP-1 cells have been used as a model system for this process (Tsuchiya *et al.*, 1980; Chanput *et al.*, 2014). Finally, cytotoxicity was assessed using cell viability assay, and the cellular uptake of ZnO NPs was estimated by raw scattering analysis using flow cytometry.

MATERIALS AND METHODS

Test substances

Two ZnO (CAS 1314-13-2) NPs with different physicochemical properties were obtained and designated as ZnO(α) (zinc oxide, NanoTek[®] ZH1121W, 40% in H₂O, colloidal, Alfa Aesar, Ward Hill, MA, USA) and ZnO(Σ) (zinc oxide, dispersion, nanoparticles, 50 wt. % in water, Sigma-Aldrich Company Ltd., New Road, Gillingham, Dorset, UK) according to their supplier information. ZnO(α) and ZnO(Σ) were suspended in distilled water (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) or RPMI 1640 culture medium. 2,4-Dinitrochlorobenzene (DNCC CAS97-00-72, Sigma-Aldrich) dissolved in DMSO was used as a positive control.

Determination of physicochemical properties

The nominal primary particle size was obtained from the supplier's data. The secondary particle size and zeta potential of ZnO(α) and ZnO(Σ) in aqueous suspensions were determined using the ELSZ-2 analyzer (Otsuka Electronics Co., Ltd., Osaka, Japan), and the average particle size (hydrodynamic particle size), polydispersity index, and particle size distribution were measured using the dynamic light scattering (DLS) method. The average particle size and polydispersity index were obtained using the cumulant method, and the particle size distribution was obtained by the histogram method using the Marquardt analysis method. The zeta potential was measured using the electrophoretic light scattering method (laser Doppler method).

Cell culture

Human monocytic leukemia cells (THP-1, ATCC TIB-202) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). THP-1 cells were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 0.055 mM 2-mercaptoethanol at 37°C with 5% CO₂. The cells were sub-cultured every three or four days to maintain a cell density between 1×10^5 and 8×10^5 cells/mL. The cultured cells were used for two weeks until two months after the start of culture.

Cell viability

Cell viability, calculated from the concentration of cellular ATP, was measured as an indicator of cytotoxicity of ZnO(α) and ZnO(Σ) in THP-1 cells at 24 and 48 hr of culture. THP-1 cells were seeded in a 96-well culture plate at a density of 2×10^4 cells/well. ZnO(α) or ZnO(Σ) suspended in the culture medium was added to the wells after 24 hr of culture, and the cells were cultured an additional 24 and 48 hr. At the end of the designated for culture period, the number of viable cells was determined as the concentration of cellular ATP using the CellTiter-Glo luminescent cell viability assay reagent (Promega Corp., Woods Hollow Road Madison, WI, USA) and a luminometer (Luminescencer-JNR AB-2100, ATTO, Tokyo, Japan) according to the manufacturer's instructions, with an incubation time of 45 or 90 min. CdSO₄ (Sigma-Aldrich) was used as the positive control. Cell viability was also determined from the proportion of dead cells stained with propidium iodide (Life Technologies, Thermo Fisher Scientific), in addition to the *in vitro* skin sensitization assay described below.

Determination of cytokines

The concentrations of Interleukin (IL)-8, Tumor Necrosis Factor (TNF)- α , IL-6, and IL-1 β in the culture medium at 24 and 48 hr were measured using a BD Cytometric Bead Array human inflammation kit (BD BioSciences, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with flow cytometry analysis. (Detection limit: IL-8, 3.6 pg/mL; TNF- α , 3.7 pg/mL; IL-6, 2.5 pg/mL; IL-1 β , 7.2 pg/mL).

Determination of cell surface antigens

Cultured cells blocked with human γ -globulin (0.01%) were incubated with fluorescein isothiocyanate-conjugated antibodies against CD54 (clone 6.5B5, 3:5 dilution, Dako, Agilent Technologies, Inc., Stevens Creek Blvd., Santa Clara, CA, USA) and CD86 (clone Fun-1, 3:5 dilution, BD PharMingen, Becton, Dickinson and Company) in fluorescence-activated cell sorter buffer (FACS buffer, PBS containing 0.1% bovine serum albumin) for 30 min at 4°C. Mouse IgG1 (clone DAK-G01, BD PharMingen) was used as an isotype control antibody. Five minutes after the addition of 2.5 μ g/mL propidium iodide (PI) for the staining of dead cells, 10,000 live cells were analyzed with a flow cytometer (FACS Calibur Cell Quest, BD BioSciences). The expression of cell surface antigens was determined as the relative fluorescence intensity (RFI) calculated from the geometric mean fluorescence intensity (MFI) according to the following formula:

$$\text{RFI (\%)} = (\text{MFI}_{\text{CT}} - \text{MFI}_{\text{IT}}) \div (\text{MFI}_{\text{CV}} - \text{MFI}_{\text{IV}}) \times 100$$

where MFI_{CT} is from cells treated with test substance and anti-CD antibody; MFI_{IT} is from cells treated with test substance and isotype control antibody; MFI_{CV} is from

cells treated with vehicle and anti-CD antibody; MFI_{IV} is from cells treated with vehicle and isotype control antibody.

Raw scattering analysis of cultured cells

THP-1 cells were analyzed using flow cytometer (FACS Calibur Cell Quest, BD BioSciences) and forward scattering (FSC) and side scattering (SSC) data were obtained for 10,000 cells simultaneously.

Statistical analysis

Lethal concentration 50% (LC_{50}), 25% (LC_{25}), and 5% (LC_5) were calculated by log-logistic curve fitting using the R software (R Core Team, 2017) with the extension package drc (Ritz *et al.*, 2015). The statistical significance of the differences between the experimental groups was examined by one-way analysis of variance with Dunnett's multiple comparison test at a probability level of 5%.

RESULTS

Physicochemical properties of ZnO(α) and ZnO(Σ)

The particle size and zeta potential of the aqueous suspensions of ZnO(α) and ZnO(Σ) are shown in Table 1 and Fig. 1. The nominal primary particle size of ZnO(α) was smaller than that of ZnO(Σ). However, in the culture medium, the secondary particle size of ZnO(α) was larger than that of ZnO(Σ). The zeta potential was negative for ZnO(α), both in culture medium and H_2O . In contrast, the zeta potential was positive for ZnO(Σ) in H_2O , but was more negative than that of ZnO(α) in the culture medium. These results suggest that ZnO(Σ) interacted with nega-

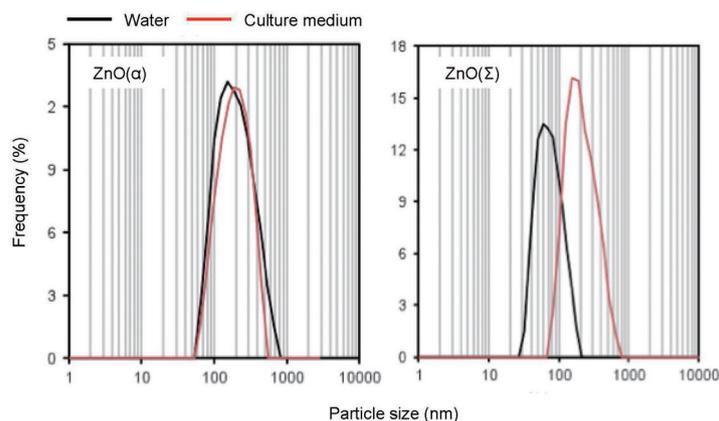


Fig. 1. Size distribution of ZnO nanoparticles in aqueous suspension. The secondary particle size of ZnO(α) and ZnO(Σ) was determined using the dynamic light scattering method. ZnO(α) and ZnO(Σ) were suspended in water or MEM culture medium at 10 mg/mL or 0.2 mg/mL, respectively.

Table 1. Particle size and zeta potential of ZnO nanoparticles.

	Nominal primary particle size (nm)	Secondary particle size (nm \pm SD)		Zeta potential (mV \pm SD)	
		10% FBS-MEM (0.2 mg/mL)	H ₂ O (10 mg/mL)	10% FBS-MEM (0.2 mg/mL)	H ₂ O (10 mg/mL)
		ZnO(α)	40	163.9 \pm 1.8	164.9 \pm 0.5
ZnO(Σ)	< 35	184.9 \pm 0.8	65.8 \pm 0.7	-13.9 \pm 0.6	44.9 \pm 0.5

The nominal primary particle size is from supplier's data. The secondary particle size and zeta potential were determined by the dynamic light scattering method and the laser doppler method, respectively.

tively charged medium constituents more efficiently than ZnO(α), possibly because of its positive zeta potential to form larger particles.

Effects of ZnO(α) and ZnO(Σ) on cytokine expression in THP-1 cells

For the cytotoxicity assay, THP-1 cells were cultured in the presence of ZnO(α) or ZnO(Σ) for 24 and 48 hr (Fig. 2A and B). The cell viability (based on the amount of cellular ATP) of THP-1 cells was analyzed to measure cytotoxicity of ZnO(α) and ZnO(Σ) at 24 and 48 hr of culture. Cell viability was decreased by ZnO(α) and ZnO(Σ) in a time- and concentration-dependent manner, and the LC₅₀ values at 24 and 48 hr were higher for ZnO(α) (24 hr, 99.6 μ g/mL; 48 hr, 53.9 μ g/mL) than for ZnO(Σ) (24 hr, 72.1 μ g/mL; 48 hr, 43.9 μ g/mL), showing greater cytotoxicity of ZnO(Σ) in THP-1 cells (Fig. 3A).

The concentrations of IL-8, TNF- α , IL-6, and IL-1 β in the culture medium of THP-1 cells at 24 and 48 hr were determined to evaluate the inductive effects of ZnO(α) and ZnO(Σ) on cytokine expression. The concentration of IL-8 was increased from 25 μ g/mL and reached about 600-fold higher levels of the control at 100 μ g/mL of ZnO(α) and ZnO(Σ), indicating strong induction of IL-8 expression (Fig. 3B). Although there was no difference between 24 and 48 hr, the response curve was shifted to the left by ZnO(α) and ZnO(Σ) based on their LC₅₀ values. TNF- α increased in a similar pattern to IL-8, but to a smaller extent, indicating weak induction of TNF- α expression (Fig. 3C). The concentrations of IL-1 β and IL-6 showed no obvious changes at 24 or 48 hr (Fig. 3D and E).

Effects of ZnO(α) and ZnO(Σ) on cell surface antigen expression in THP-1 cells

Cytotoxicity was determined with narrower concentration and replicated data sets at 24 and 48 hr culture; 12.5 μ g/mL was the no effect concentration for both ZnO(α) and ZnO(Σ), confirming the presence of a threshold (Fig. 4A, left and middle). The LC₅ value at 24 hr and LC₂₅ value at 48 hr, which could be obtained within the

concentration range, was calculated at 36.5 μ g/mL and 42.7 μ g/mL for ZnO(α), and at 26.7 μ g/mL and 30.0 μ g/mL for ZnO(Σ), respectively, confirming the greater cytotoxic effect of ZnO(Σ) and the left-shift in its response curve. DNCB, a positive control chemical that induces the expression of CD cell surface antigens, decreased cell viability at lower concentrations than those induced by ZnO(α) or ZnO(Σ). However, the cell viability was above 50% throughout the concentration range, and the LC₅ value at 24 hr and LC₂₅ value at 48 hr were calculated at 1.88 μ g/mL and 2.97 μ g/mL, respectively (Fig. 4A, right).

CD54 expression in THP-1 cells was significantly increased by both ZnO(α) and ZnO(Σ), greater than 10-fold of the control level in a concentration-dependent manner at cytotoxic concentrations, but there was no difference found between 24 and 48 hr (Fig 4B, left and middle). Further, the increase in CD54 expression was smaller for ZnO(α) than for ZnO(Σ), but was similar when compared at cytotoxic concentrations (see LC₂₅ and LC₅ values in Fig. 4A). Therefore, this finding suggests that ZnO(α) and ZnO(Σ) caused the same responses, probably due to cellular uptake instead of the concentration in the culture medium. DNCB increased CD54 expression approximately 5-fold at 24 hr compared to approximately 2-fold at 48 hr, indicating its more potent inductive effects at shorter exposure times (Fig. 4B, right). However, CD54 expression induced by DNCB was lower than that by ZnO(α) or ZnO(Σ), even when compared at LC₂₅ and LC₅.

CD86 expression in THP-1 cells cultured in the presence of ZnO(α) or ZnO(Σ) showed a U-shaped response, such that expression levels were lower at low concentrations (Fig. 4C, left and middle). Similar to the effects of ZnO(α) or ZnO(Σ) on CD54 expression, there were no differences in CD86 expression between 24 and 48 hr. However, there was no increase in CD86 expression by ZnO(α) and ZnO(Σ). On the other hand, DNCB increased CD86 expression by approximately 2.5-fold, indicating its inductive effects (Fig. 4C, right).

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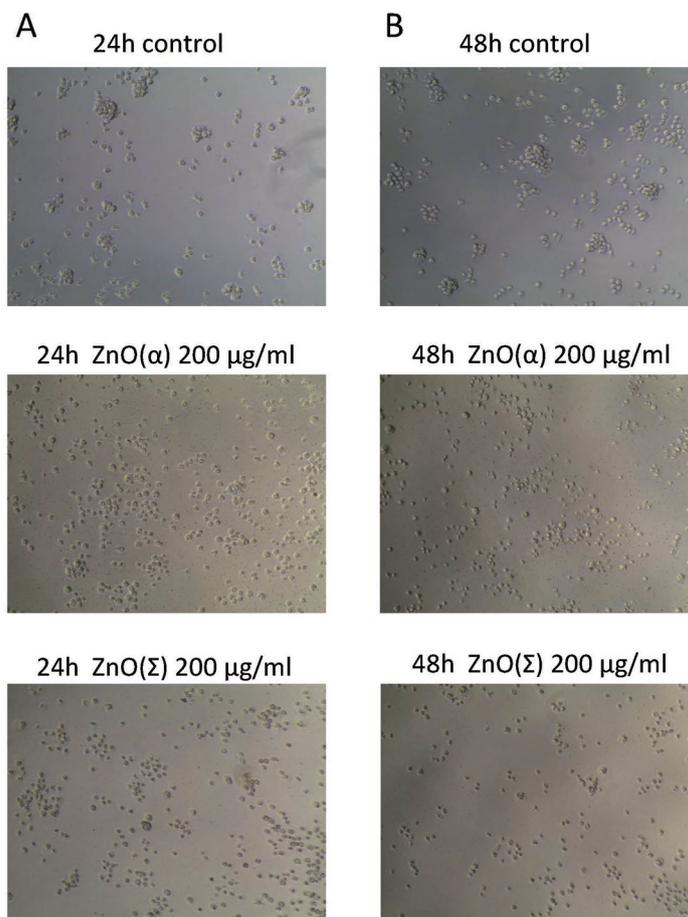


Fig. 2. THP-1 cells cultured in the presence of ZnO(α) or ZnO(Σ). THP-1 cells were seeded in a 96-well culture plate at 2×10^4 cells/well. Subsequently, ZnO(α) or ZnO(Σ) suspended in culture medium was added to the well after 24 hr of culture, and the cells were cultured for an additional 24 hr (A) and 48 hr (B).

Scattering analysis of THP-1 cells exposed to ZnO(α) and ZnO(Σ)

To estimate ZnO(α) and ZnO(Σ) uptake into THP-1 cells, FSC and SSC were analyzed by flow cytometry (Fig. 5). There were no obvious changes in FSC due to ZnO(α) and ZnO(Σ), indicating no changes in the overall cell appearance. However, SSC was increased by ZnO(α) and ZnO(Σ) in a concentration-dependent manner, with a greater increase for ZnO(Σ), indicating greater cellular uptake.

DISCUSSION

These findings indicate that ZnO NPs induced the expression of immune cell surface antigen CD54, proinflammatory cytokines IL-8 and TNF- α , and their associat-

ed immunomodulatory activities. Two ZnO NPs, ZnO(α) and ZnO(Σ), with different physicochemical properties, showed the same immunomodulatory activities with varied potencies. However, their potencies were comparable when normalized to their cytotoxic concentrations, such as LC₅₀ and LC₂₅. It is thus considered that there is a potency shift that is associated with cytotoxicity and physicochemical properties, in immunomodulatory activities in THP-1 cells between ZnO NPs.

This potency shift is significant in the selection and design of ZnO NPs based on their immunomodulatory activities because the relative immunomodulatory activities of ZnO NPs can be predicted easily from their cytotoxicity, owing to this potency shift under experimental conditions as observed in the present study. It is thus expected that this potency shift will facilitate the develop-

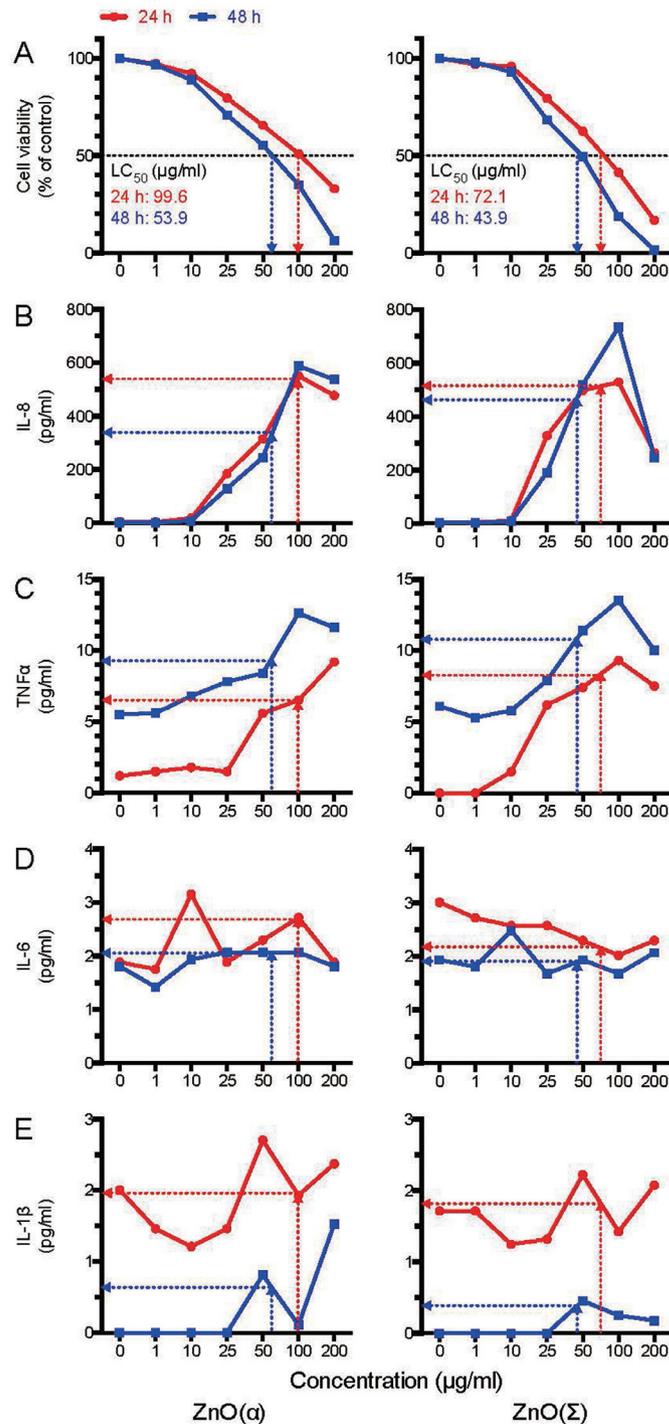


Fig. 3. Cytotoxicity and cytokine expression in THP-1 cells cultured in the presence of ZnO(α) or ZnO(Σ). THP-1 cells were cultured for 24 or 48 hr. Values represent mean \pm SEM, $n = 2$. (A) Cytotoxicity determined as cell viability. Cell viability was calculated by measuring the concentration of cellular ATP. Lethal concentration 50% (LC₅₀) was calculated by log-logistic curve fitting. Confidence limits (95%) of the LC₅₀ values were 92.3–106.9 $\mu\text{g}/\text{mL}$ at 24 hr and 41.7–66.0 $\mu\text{g}/\text{mL}$ at 48 hr for ZnO(α), and 64.0–80.2 $\mu\text{g}/\text{mL}$ at 24 hr and 36.8–51.2 $\mu\text{g}/\text{mL}$ at 48 hr for ZnO(Σ). (B–E) Expressions of IL-8, TNF- α , IL-6, and IL-1 β . Cytokine concentrations in the culture medium were determined using the BD Cytometric Bead Array (CBA) human inflammation kit. Dotted arrows indicate the LC₅₀ values and corresponding cytokine expression levels.

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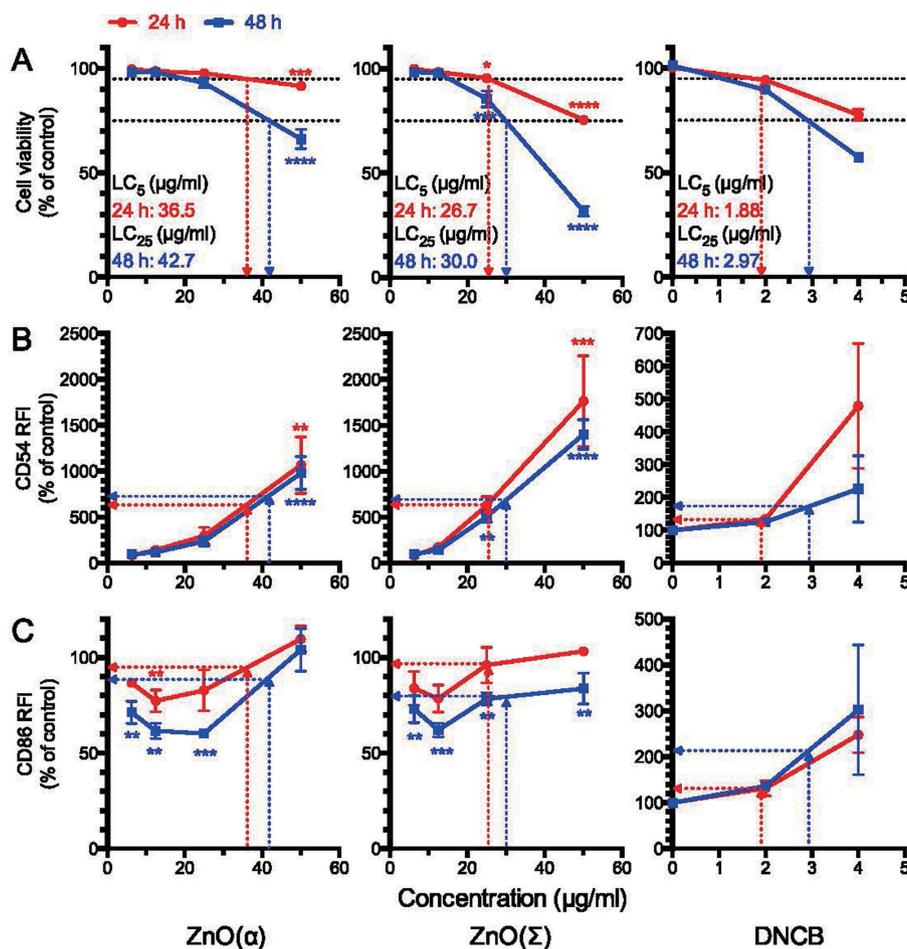


Fig. 4. Cytotoxicity and cell surface antigen expression in THP-1 cells cultured in the presence of ZnO(α) or ZnO(Σ). THP-1 cells were cultured for 24 or 48 hr. Values represent mean \pm SEM, $n = 3$. 2,4-Dinitrochlorobenzene (DNCB) was used as a positive control. Asterisks indicate statistically significant differences compared to 0 $\mu\text{g}/\text{mL}$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (A) Cytotoxicity determined as cell viability. Cell viability was calculated by measuring the concentration of cellular ATP. Lethal concentration 25% (LC_{25}) and 5% (LC_5) were calculated by log-logistic curve fitting. Confidence limits (95%) of the LC_5 and LC_{25} values were 29.3–43.7 $\mu\text{g}/\text{mL}$ and 38.8–46.6 $\mu\text{g}/\text{mL}$ for ZnO(α), 0.66–52.8 $\mu\text{g}/\text{mL}$ and 27.3–32.7 $\mu\text{g}/\text{mL}$ for ZnO(Σ), and 1.11–2.65 $\mu\text{g}/\text{mL}$ and 2.01–3.93 $\mu\text{g}/\text{mL}$ for DNCB, respectively. (B, C) Expressions of cell surface antigens CD54 and 86. Cell surface antigen expression in 10,000 viable cells was measured using the relative fluorescence intensity (RFI). Dotted arrows indicate the LC values and corresponding cell surface antigen expression levels.

ment of industrial products and biomedical applications that require the control of immunomodulatory activities of ZnO NPs.

Further, this study suggests that the amount of ZnO(α) and ZnO(Σ) taken up by THP-1 cells is related to the potency shift in immunomodulatory activities. This is because ZnO(Σ), which is taken up in greater amounts into the cells as estimated by the SSC analysis, induced more potent immunomodulatory responses. Therefore, this finding suggests that the differing physicochemical properties between ZnO(α) and ZnO(Σ) impact cellu-

lar uptake and are important for their immunomodulatory activities.

Among the physicochemical properties determined in this study, the secondary particle size and zeta potential are known to be involved in the cellular uptake of NPs. For example, ZnO NPs with a larger secondary particle size are taken up by THP-1 cells via phagocytosis (Sahu *et al.*, 2014). Silicon dioxide NPs are taken up into colorectal adenocarcinoma CaCo-2 cells when aggregated to approximately 120 nm in the culture medium (Halamoda-Kenzaoui *et al.*, 2017). In the same cell line, negative-

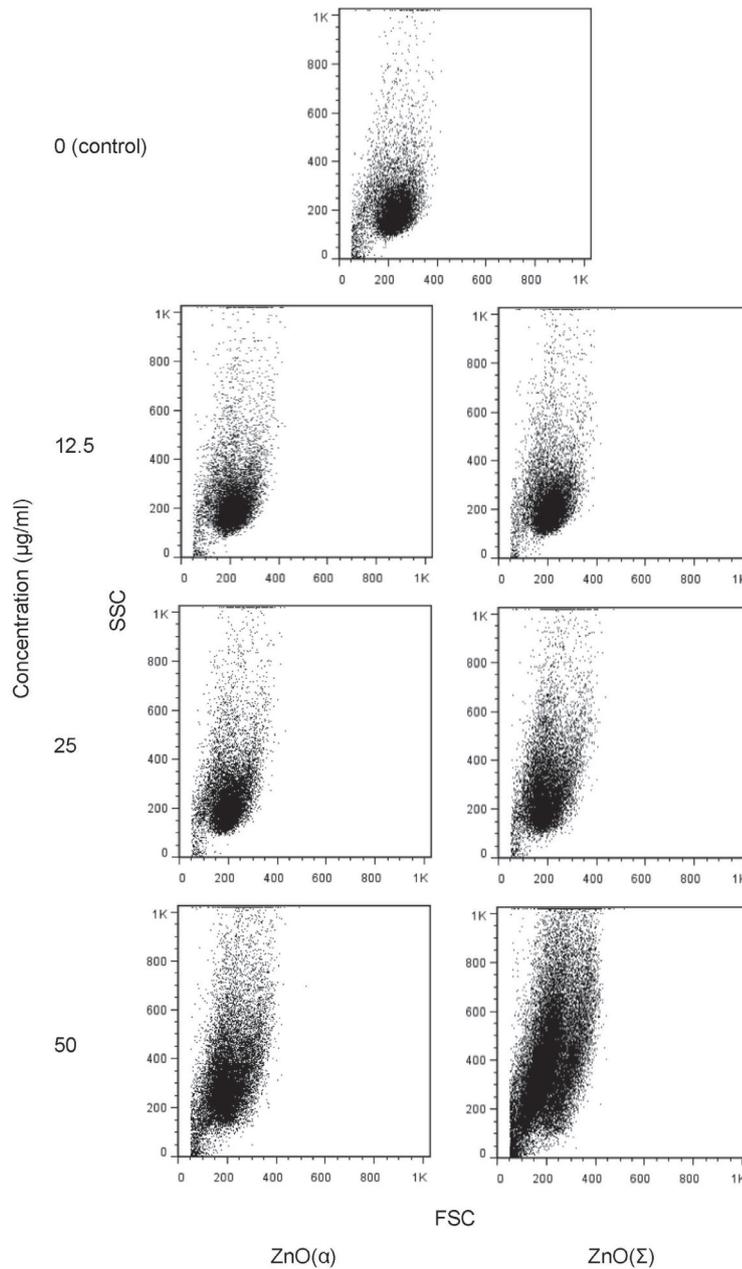


Fig. 5. Forward scatter (FSC) versus side scatter (SSC) density plot of THP-1 cells cultured in the presence of ZnO(α) or ZnO(Σ). THP-1 cells were cultured for 24 hr. FSC and SSC were analyzed by flow cytometry for 10,000 viable cells.

ly charged nanoparticles are more efficiently taken up than their positively charged counterparts (Bannunah *et al.*, 2014). These findings are consistent with this study because ZnO(Σ), which is larger in secondary particle size and more negatively charged in zeta potential than ZnO(α) in the culture medium, was taken up into THP-1

cells more efficiently.

The relationship between the primary particle size and the cytotoxicity and immunomodulatory activities of ZnO NPs in this study is also consistent with previous reports (Sahu *et al.*, 2014). It has been shown that ZnO NPs with a smaller primary particle size decreased cell viability and

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stimulated the release of cytokines, such as TNF- α , IL-6, and IL-1 β , in THP-1 cells more potently than those with a larger primary particle size (Sahu *et al.*, 2014; Feltis *et al.*, 2012). Consistent with these reports, the more potent cytotoxicity of ZnO(Σ) in THP-1 cells may be at least partly due to its primary particle size being smaller than that of ZnO(α).

From a toxicological perspective, this study is consistent with the *in vitro* skin sensitization test by OECD, where double-positive results for the induction of CD54 and CD86 expression at LC₂₅ values in THP-1 cells were judged as possible sensitizer activity (OECD, 2016). Based on these criteria, both ZnO(α) and ZnO(Σ) are considered probable non-skin sensitizers, because the induction of CD54 expression was not found in this study.

Although mechanistic investigations were not conducted in this study, the mechanisms of cytotoxicity and immunomodulatory activities of ZnO NPs differ because the cytotoxicity of ZnO(α) and ZnO(Σ) is time-dependent, while their immunomodulatory activities are not, as shown by the differences between 24 and 48 hr of culture. However, it is not known whether the cytotoxicity and immunomodulatory activities of ZnO NPs are mechanistically independent, as has been previously proposed in THP-1 cells (Feltis *et al.*, 2012).

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Aiba, S., Terunuma, A., Manome, H. and Tagami, H. (1997): Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol.*, **27**, 3031-3038.
- Bannunah, A.M., Vllasaliu, D., Lord, J. and Stolnik, S. (2014): Mechanisms of nanoparticle internalization and transport across an intestinal epithelial cell model: effect of size and surface charge. *Mol. Pharm.*, **11**, 4363-4373.
- Chanput, W., Mes, J.J. and Wichers, H.J. (2014): THP-1 cell line: an *in vitro* cell model for immune modulation approach. *Int. Immunopharmacol.*, **23**, 37-45.
- Dukhinova, M.S., Prilepskii, A.Y., Shtil, A.A. and Vinogradov, V.V. (2019): Metal oxide nanoparticles in therapeutic regulation of macrophage functions. *Nanomaterials (Basel)* **9**, 1631.
- Feltis, B.N., O'Keefe, S.J., Harford, A.J., Piva, T.J., Turney, T.W. and Wright, P.F. (2012): Independent cytotoxic and inflammatory responses to zinc oxide nanoparticles in human monocytes and macrophages. *Nanotoxicology*, **6**, 757-765.
- Ha, N.Y., Shin, H.M., Sharma, P., Cho, H.A., Min, C.K., Kim, H., Yen, N.T., Kang, J.S., Kim, I.S., Choi, M.S., Kim, Y.K. and Cho, N.H. (2016): Generation of protective immunity against *Orientia tsutsugamushi* infection by immunization with a zinc oxide nanoparticle combined with ScaA antigen. *J. Nanobiotechnology*, **14**, 76.
- Halamoda-Kenzaoui, B., Ceridono, M., Urbán, P., Bogni, A., Ponti, J., Gioria, S. and Kinsner-Ovaskainen, A. (2017): The agglomeration state of nanoparticles can influence the mechanism of their cellular internalisation. *J. Nanobiotechnology*, **15**, 48.
- Hanley, C., Thurber, A., Hanna, C., Punnoose, A., Zhang, J. and Wingett, D.G. (2009): The influences of cell type and ZnO nanoparticle size on immune cell cytotoxicity and cytokine induction. *Nanoscale Res. Lett.*, **4**, 1409-1420.
- Jatana, S., Palmer, B.C., Phelan, S.J. and Delouise, L.A. (2017): Immunomodulatory effects of nanoparticles on skin allergy. *Sci. Rep.*, **7**, 3979.
- Jiang, J., Pi, J. and Cai, J. (2018): The advancing of zinc oxide nanoparticles for biomedical applications. *Bioinorg. Chem. Appl.*, **2018**, 1-18.
- Kim, K.B., Kim, Y.W., Lim, S.K., Roh, T.H., Bang, D.Y., Choi, S.M., Lim, D.S., Kim, Y.J., Baek, S.H., Kim, M.K., Seo, H.S., Kim, M.H., Kim, H.S., Lee, J.Y., Kacew, S. and Lee, B.M. (2017): Risk assessment of zinc oxide, a cosmetic ingredient used as a UV filter of sunscreens. *J. Toxicol. Environ. Heal. Part B. Crit. Rev.*, **20**, 155-182.
- OECD. (2016): Test No. 442E: *In vitro* skin sensitisation. In: OECD guidelines for the testing of chemicals, Section 4, pp.1-21, OECD Publishing.
- R Core Team. (2017): R: A language and environment for statistical computing. Vienna, Austria. <http://www.R-project.org/>
- Ritz, C., Baty, F., Streibig, J.C. and Gerhard, D. (2015): Dose-response analysis using R. *PLoS One*, **10**, e0146021.
- Roy, R., Kumar, S., Verma, A.K., Sharma, A., Chaudhari, B.P., Tripathi, A., Das, M. and Dwivedi, P.D. (2013): Zinc oxide nanoparticles provide an adjuvant effect to ovalbumin via a Th2 response in Balb/c mice. *Int. Immunol.*, **26**, 159-172.
- Sahu, D., Kannan, G.M. and Vijayaraghavan, R. (2014): Size-dependent effect of zinc oxide on toxicity and inflammatory potential of human monocytes. *J. Toxicol. Environ. Heal. Part A Curr. Issues*, **77**, 177-191.
- Saptarshi, S.R., Feltis, B.N., Wright, P. and Lopata, A.L. (2015): Investigating the immunomodulatory nature of zinc oxide nanoparticles at sub-cytotoxic levels *in vitro* and after intranasal instillation *in vivo*. *J. Nanobiotechnology*, **13**, 6.
- Senapati, V.A., Kumar, A., Gupta, G.S., Pandey, A.K. and Dhawan, A. (2015): ZnO nanoparticles induced inflammatory response and genotoxicity in human blood cells: A mechanistic approach. *Food Chem. Toxicol.*, **85**, 61-70.
- Singh, S. (2019): Zinc oxide nanoparticles impacts: cytotoxicity, genotoxicity, developmental toxicity, and neurotoxicity. *Toxicol. Mech. Methods*, **29**, 300-311.
- Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980): Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer*, **26**, 171-176.
- Vujovic, M. and Kostic, E. (2019): Titanium dioxide and zinc oxide nanoparticles in sunscreens: a review of toxicological data. *J. Cosmet. Sci.*, **70**, 223-234.