

ORIGINAL ARTICLE

Adjuvant effect of zinc oxide on Th2 but not Th1 immune responses in mice

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Abstract

Background and aim: We investigated the effect of zinc oxide (ZnO) on Th1 and Th2 immune responses in mice.

Material and methods: Mice were intraperitoneally administered with ovalbumin (OVA) with or without varying doses of ZnO (day 0). On day 21, anti-OVA IgG, IgG2a, IgG1, and IgE antibodies in sera, OVA-specific proliferative responses of spleen cells, and production of Th1 cytokines including IFN- γ as well as Th2 cytokines such as IL-4 and IL-5 were measured.

Results: The results showed that administration of OVA with ZnO was followed by greater increases in anti-OVA IgG and the antigen-specific splenocyte proliferation compared to that of OVA alone. The production of anti-OVA IgG1 and IgE and secretion of IL-4 and IL-5 were markedly enhanced by ZnO. The enhancing effect of ZnO on these Th2 responses was as strong as aluminium hydroxide (Alum) that was widely used as an adjuvant. In contrast, treatment with OVA plus ZnO failed to affect production of anti-OVA IgG2a as well as IFN- γ . It was also observed that ZnO had a stimulating effect on the secretion of the proinflammatory cytokine IL-17 from a new lineage of effector Th cells.

Conclusion: These results suggest that ZnO appears to have an adjuvant effect on the immune system, especially Th2 but not Th1 immune responses.

Keywords: Zinc oxide; adjuvant; Th1; Th2; antibody; cytokine

Introduction

Zinc oxide (ZnO) is available as a dietary supplement and generally recognized as a safe compound in the most countries. Previous toxicological studies demonstrated that the LD₅₀ of ZnO in rats was 5 g/kg and the compound was shown to be non-toxic when it was given orally. Therefore, ZnO is categorized as a non-toxic chemical according to the Globally Harmonized Classification System for the classification of chemicals[1]. There have been several studies suggesting that the excess of Zn²⁺ consumption through the diet[1] does not cause any adverse effects in humans.

ZnO has also been used in personal care products including makeup, nail products, baby lotions, bath soaps, foot powders and over-the-counter (OTC) drug products for many years. Nevertheless, there appears to

be possibility that ZnO might be absorbed via not only digestive organs but also other tissues such as airways and the skin following its use[2,3].

There are also some studies demonstrating that ZnO is biologically active. For instance, ZnO has an ability to enhance the cellular uptake of anticancer drugs in a leukemia cell line[4]. ZnO has also been shown to be toxic for both gram-negative and gram-positive bacteria[5]. Therefore, more detailed investigation appears to be required in terms of the safety of ZnO.

CD4⁺ T cells are mainly classified into two subsets, types I (Th1) and II (Th2), on the basis of their distinct cytokine profiles[6]. Th1 cells predominately secrete Th1 cytokines such as IL-2 and IFN- γ involved in cellular immunity, whereas Th2 cells produce cytokines including IL-4 and IL-5 playing a role in humoral immunity. A number of studies demonstrated that Th1 and Th2

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immune responses are modulated by various environmental factors[7]. Recently, Th17 cells have been identified as a new lineage of effector Th cells and have been shown to play an important role in immune responses to infectious agents as well as in various immune diseases[8,9].

In the present study, we show that ZnO had an ability to enhance immune responses, especially Th2 but not Th1 response. ZnO also enhanced the secretion of Th17 from spleen cells. The adjuvant effect of ZnO was as strong as aluminium hydroxide (Alum) that was frequently used as an adjuvant.

Materials and Methods

Animals

Male 8- to 9-week-old DBA/1J mice were used in all experiments. The mice were purchased from Charles River Laboratories Japan, Inc. (Shiga, Japan). They were maintained in a temperature- and light-controlled environment with free access to standard rodent chow and water. The experiments were performed according to the Ethical Guidelines of Shionogi.

Administration of antigen and ZnO

Ovalbumin (OVA, Sigma Chemical Co., St Louis, MO, USA) was dissolved in phosphate buffered saline (PBS). Varying doses (1.2, 4, and 12 mg/mL) of ZnO (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) were suspended in PBS. ZnO suspension and the antigen solution were mixed together in equal volumes using a vortex mixer for several seconds.

Mice were then immediately immunized by intraperitoneal injections of 500 μ L of PBS containing varying doses (0.3, 1, and 3 mg) of ZnO and/or OVA (100 μ g) on day 0. Imject Alum (Alum, Thermo Fisher Scientific Inc., Rockland, IL, USA), a commonly used adjuvant, was used as a positive control. The antigen solution was added to Alum dropwise with constant mixing at a 1:1 volume ratio of Alum to antigen. After continuous mixing for 30 minutes, 500 μ L of Alum/antigen solution was given intraperitoneally. Each treatment group consisted of 5 to 6 mice.

Measurement of OVA-specific antibodies

On day 21 after immunization, mice were anesthetized with ether and blood was collected by cardiac puncture. After blood collection, the animals were euthanized by cervical dislocation. The blood samples were collected in SEPACLEAN A-5 tubes (Eiken Kizai Co., Ltd., Tokyo, Japan) and were centrifuged to separate the serum,

which was stored at -20°C until analysis. IgG, IgG1, IgG2a, and IgE antibodies specific for OVA were individually measured using an ELISA.

For the measurement of IgG, IgG1, and IgG2a, 96-well flat-bottomed microtiter plates were incubated with 100 μ L well $^{-1}$ of OVA (100 μ g mL $^{-1}$) at 4°C overnight and washed three times with PBS. The wells were then blocked by incubation with 200 μ L of PBS containing 1% casein (Sigma-Aldrich Co.) at 37°C for 1 h. After washing, dedicated wells received 100 μ L of 1:100000 (for IgG measurement), 1:1000 (for IgG1 measurement), or 1:100 (for IgG2a measurement) dilutions of each serum sample, and the plates were then incubated at 37°C for 1 h. The plates were then washed, and 100 μ L well $^{-1}$ of a 1:1000 dilution of goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich Co.), rat anti-mouse IgG1 or IgG2a labeled with alkaline phosphatase (BD Pharmingen, San Diego, CA, USA) were added to wells corresponding to the primary antibody target and incubated at 37°C for 1 h. After washing, 100 μ L of 3 mM *p*-nitrophenyl phosphate (Bio-Rad Laboratories, Hercules, CA, USA) well $^{-1}$ was added and the plates were incubated in the dark at room temperature until the bright yellow color of the reaction product was obtained. The absorbance was then measured at 405 nm using a VersamMax microplate reader (MDS Inc., Ontario, Canada) (Sunnyvale, CA, USA). The results were expressed as absorbance units at $\text{OD}_{405} \pm \text{S.E.M.}$

For the measurement of IgE, 96-well flat-bottomed microtiter plates were incubated with 100 μ L of 1 μ g mL $^{-1}$ rat anti-mouse IgE (MONOSAN, Uden, The Netherlands) in each well at 37°C for 1 h and washed three times with SuperBlock Blocking Buffer (Thermo Fisher Scientific, Inc.). The plates were incubated with 100 μ L of a 1:5 dilution of each serum sample at 37°C for 1 h.

The plates were washed with PBS containing Tween 20 detergent at a concentration of 0.05% and 100 μ L well $^{-1}$ of 1 μ g mL $^{-1}$ biotinylated OVA was added and incubated at 37°C for 1 h. After washing, 100 μ L of 1 μ g mL $^{-1}$ of streptavidin conjugated with horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) well $^{-1}$ was added per well and incubated at 37°C for 1 h. After washing, 100 μ L of the TMB substrate solution (Thermo Fisher Scientific, Inc.) was added to each microplate well and the plates were incubated at room temperature for 30 min. The reaction was stopped by adding 100 μ L of 2 M sulfuric acid to each well. The absorbance was then measured at 450 nm using a VersamMax microplate reader (MDS Inc.). The results were expressed as absorbance units at $\text{OD}_{450} \pm \text{S.E.M.}$

Proliferation assay

Spleens were removed on day 21 and cell suspensions prepared[10]. Erythrocytes in the cells were

lysed with Tris-NH₄Cl. A total of 5×10^5 cells in 0.1 mL of RPMI 1640C (RPMI 1640 [SIGMA] containing 1 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 µg streptomycin, 4×10^{-5} M 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum) were cultured in each microwell in the presence or absence of 500 µg mL⁻¹ of OVA. After 48 h, each well was pulsed with 0.5 µCi of tritium thymidine and the cells were cultured for another 18 h. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques. Results, expressed in cpm, are the average of triplicate cultures of cells pooled from 5 to 6 mice.

Measurement of cytokines

One milliliter of RPMI 1640C containing a total of 5×10^6 spleen cells was cultured in each well of 24-well tissue culture plates in medium alone or with 100 µg mL⁻¹ or 500 µg mL⁻¹ of OVA. After 4 days, the supernatants were harvested and stored at -20°C until assayed. Secretion of IFN-γ, IL-5, IL-4 and IL-17 was quantified using ELISA kits (Endogen, Inc., Woburn, MA, USA for IFN-γ, IL-5, and IL-4 measurement; R&D Systems Inc., Minneapolis, MN, USA for IL-17 measurement).

Statistics

To analyze data statistically, the Dunnett's parametric multiple t-test was used to compare each ZnO or Alum groups with OVA/PBS groups.

Results

Effect of ZnO on the production of anti-OVA IgG antibodies and proliferative responses of spleen cells to OVA

To examine the effect of ZnO on the production of antibodies to OVA, OVA was given i.p. with 0.3, 1 and 3 mg of ZnO (day 0) followed by measurement of anti-OVA IgG antibodies in sera on day 21. As shown in Figure 1, administration of ZnO plus OVA enhanced anti-OVA IgG production in a dose-related fashion, whereas antibody production was barely observed in mice treated with OVA alone.

The serum level of anti-OVA IgG in mice given OVA with the highest dose of ZnO was six folds higher than that in animals administered with OVA alone. The enhancing effect of OVA plus 3 mg of ZnO on the antibody production was similar to that of OVA plus Alum. Proliferative responses to OVA of spleen cells also tended to be greater, but only in mice injected with the highest ZnO plus OVA group did this value approach statistical significance (i.e., $p = 0.0067$) (Table 1).

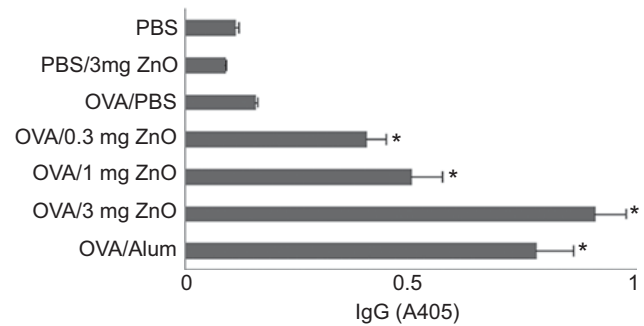


Figure 1. Effect of ZnO on anti-OVA IgG antibody production. Mice were i.p. injected with 100 µg of OVA with or without 0.3, 1, and 3 mg of ZnO or aluminium hydroxide (day 0). PBS or 3 mg of ZnO alone was given as a control. On day 21, anti-OVA IgG antibodies in sera were determined by ELISA as shown in Materials and Methods. Values are expressed as mean ± S.E.M. of 5 to 6 mice. * $P < .05$ vs. OVA/PBS (Dunnett's test).

Table 1. Effect of ZnO on proliferation (cpm; $n=3$) of spleen cells to OVA. Mice were i.p. injected with 100 µg of OVA with or without 0.3, 1, and 3 mg of ZnO or aluminium hydroxide (day 0). On day 21, spleens were removed, cells isolated, pooled, and then incubated with 500 µg OVA/ml to permit measurement of the proliferative capacity (as outlined in Methods). Values shown are the mean ± SE of triplicate cultures/cohort.

ZnO (mg/animal)	Proliferation (c.p.m)
0 (PBS)	16,123 ± 1333
0.3	15,975 ± 827
1	21,110 ± 2075
3	20,937 ± 868*

* $P < .05$ vs. OVA/PBS (Dunnett's test).

Effect of ZnO on the production of anti-OVA IgG1, IgE and IgG2a antibodies

The effect of ZnO on potential Th1 and Th2 immune responses was also investigated here. The results shown in Figure 2 indicate that administration of OVA with ZnO resulted in a significant increase in production of anti-OVA IgG1 at all doses of the ZnO tested. In contrast, the formation of anti-OVA IgE, a process in which Th2 cells were involved, was only significantly increased at the dose of 3 mg ZnO plus OVA. In no cases were there any significant effects of the ZnO on the production of anti-OVA IgG2a, a process that was dependent on Th1 cells.

Effect of ZnO on the secretion of cytokines

To investigate the effect of ZnO on the secretion of cytokines, IFN-γ (Th1 cytokines), IL-4 and IL-5 (Th2 cytokines) from spleen cells were measured. As shown in Figure 3, administration of OVA plus ZnO failed to affect IFN-γ production.

In contrast, the secretion of IL-5 when cells were incubated with OVA tended to be enhanced in the mice that had received the ZnO plus OVA treatments. With

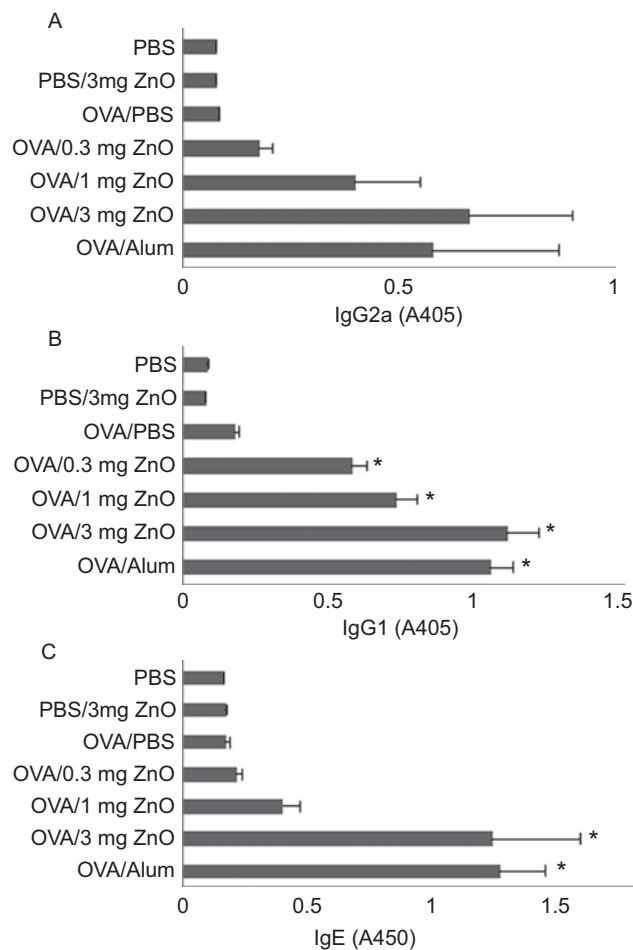


Figure 2. Effect of ZnO on anti-OVA IgG2a, IgG1, and IgE antibody production. Mice were i.p. injected with 100 μ g of OVA with or without 0.3, 1, and 3 mg of ZnO or aluminium hydroxide (day 0). PBS or 3 mg of ZnO alone was given as a control. On day 21, anti-OVA IgG2a, IgG1, and IgE antibodies in sera were determined by ELISA as shown in Materials and Methods. Values are expressed as mean $\pm$ S.E.M. of 5 to 6 mice. * $P < .05$ vs. OVA/PBS (Dunnett's test).

respect to IL-4, only the co-treatment of 3 mg ZnO plus OVA appeared to have yielded cells that evinced a strong degree of production in response to the added OVA. This same pattern of response was also evident with regard to the OVA-stimulated production of IL-17 by the pooled spleen cells from each cohort (Figure 4). A similar effect on the secretion of these latter three cytokines was observed when mice had been given a combination of OVA and Alum 21 days before their spleen cells were harvested for analyses.

Discussion

The present study demonstrates that ZnO may have an adjuvant effect on the immune system since treatment of mice with a combination of OVA and ZnO appeared to

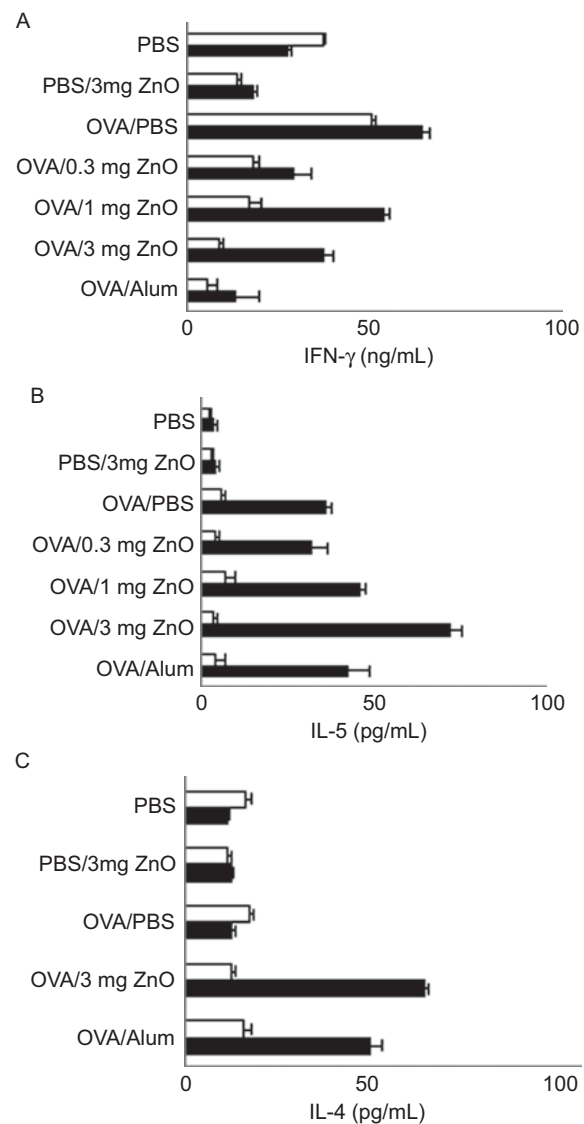


Figure 3. Effect of ZnO on IFN- γ , IL-5, and IL-4 production. Mice were i.p. injected with 100 μ g of OVA with or without 0.3, 1, and 3 mg of ZnO or aluminium hydroxide (day 0). PBS or 3 mg of ZnO alone was given as a control. On day 21, spleens were removed and spleen cells were incubated with (solid columns) or without (open columns) 100 μ g/ml of OVA followed by measurement of IFN- γ (A) and IL-5 (B) in the culture supernatant as shown in Materials and Methods. IL-4 (C) was also measured on day 7. Values are shown as mean $\pm$ S.E.M. of triplicate samples from culture supernatants of cell pooled from 5 to 6 mice.

augment the production of OVA-specific IgG and, when "high" levels of ZnO were used in this combination, the antigen-specific proliferative responses of splenocytes. Few previous studies directly demonstrated that ZnO had such adjuvant effects on immune responses.

However, it has been shown that ZnO had stimulating effects on the secretion of inflammatory cytokines. Specifically, it was also shown that treatment with ZnO was followed by the production of TNF- α and IL-8

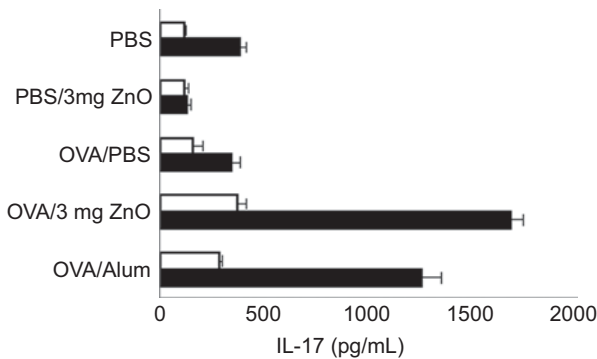


Figure 4. Effect of ZnO on IL-17 production. Mice were i.p. injected with 100 μ g of OVA with or without 3mg of ZnO or aluminium hydroxide (day 0). PBS or 3mg of ZnO alone was given as a control. On day 21, spleens were removed and spleen cells were incubated with (solid columns) or without (open columns) 500 μ g/ml of OVA followed by measurement of IL-17 in the culture supernatant as shown in Materials and Methods. Values are shown as mean $\pm$ S.E.M. of triplicate samples from culture supernatants of cell pooled from 5 to 6 mice.

by U937 human mononuclear cells[11] as well as the expressions of IL-8 and monocyte chemotactic protein-1 mRNA in human aortic endothelial cells[12]. However, these in vitro results do not directly indicate that ZnO has an ability to modulate the in a living host immune system.

Our studies also demonstrate that treatment with OVA plus ZnO was associated with increases in anti-OVA IgG1 and IgE but not IgG2a. Since IgG1 and IgE production is dependent on Th2 cells and IgG2a on Th1 cells[13,14], the increases in these antibodies in ZnO-treated mice suggest that ZnO appears to have an adjuvant effect on Th2 but not Th1 responses. This was also supported by the result that the secretion of the Th2 cytokines IL-4 and IL-5 (but not the Th1 cytokine IFN- γ) appeared to be augmented by ZnO.

It is well known that Alum is used as an adjuvant and markedly induces Th2 but not Th1 responses[15,16], although recent studies demonstrated the adjuvant effect of Alum was mediated by inflammasomes[17]. For instance, administration of Alum with antigen results in the production of antigen-specific IgG1 as well as the secretion of IL-4 and IL-5 but not IgG2a and IFN- γ production[15]. Since a similar adjuvant effect was seen in ZnO-treated mice, Alum and ZnO appear to share the ability to modulate the Th2 but not Th1 immune system. The precise mechanism by which exposure to ZnO resulted in augmentation of Th2 immune reactions is unknown at present.

However, one of possible mechanisms might be that a small amount of Zn²⁺ released from the ZnO[18] (solubility of ZnO: 1.6 mg/L) provided may in part play a role in the augmentation of Th2 responses since it was previously reported that T cells markedly produced IL-4

following their stimulation by concanavalin A[19] when Zn²⁺ was present.

Zinc is an essential nutritional component required for normal development and the maintenance of immune function[20]. It was also shown that zinc-deficient mice exhibited reduced natural killer cell-mediated cytotoxic activity[21], antibody-mediated responses[22], and host defense against pathogens[22] and tumors[23]. Interestingly, zinc-deficiency causes the inhibition of IL-4 production in mice infected with *Heligmosomoides polygyrus* in which Th2 responses are preferentially produced compared to Th1 responses[24].

In addition, the levels of IL-5, parasite-specific IgG1, and IgE are also lower in the zinc-deficient mice. Moreover, the suppression of Th2 responses in zinc-deficiency is due to impaired APC and T cell functions[25]. These results suggest that zinc in ZnO might have specifically upregulated Th2 immune reactions via modification of APC and T cell functions in mice treated with the compound.

In addition to Th1 and Th2 cells, a third subset of effector Th cells has recently been described and termed Th17 cells. Th17 cells produce IL-17 and play an important role in inflammation, autoimmune diseases, cancers, and infections[26]. Our results demonstrated that ZnO appeared to stimulate Th17 differentiation since IL-17 was produced in mice given both OVA and ZnO. Since it is known that IL-6 initiates Th17 differentiation when transforming growth factor- β is present[27-29], ZnO might have differentiated Th17 cells through production of IL-6[30].

ZnO might also have modulated the cellular functions due to the increase in intracellular Zn²⁺, since Zn²⁺ acts as a second messenger. For instance, recent studies showed that extracellular stimuli such as high affinity IgE receptor (Fc ϵ receptor I [Fc ϵ RI]) cross-linking directly induced a release of free zinc from the area of the ER in mast cells, a phenomenon called the zinc wave[31]. Thus, free zinc might be taken up into the cytoplasm and modulate the functions of various types of cells including Th2 cells after administration of a combination of OVA and ZnO.

Alternatively, the enhancement of Th2 responses by ZnO may be also in part explained by the size of the compound. Particles with a diameter less than 10 μ m have been reported to have an adjuvant effect on the production of allergen-specific IgE in mice[32,33]. Polymethymethacrylate (1 μ m diameter spheres) alters the Th1/Th2 balance in mice while larger sized particles had less or no effect on the immune system[32]. Since the diameter of ZnO used in our studies was 21 nm, the adjuvant effect of ZnO observed may be in part due to the nanosize of the compound.

Another possible explanation of the adjuvant effect of ZnO is that ZnO mixed with OVA before its injection might have caused changes of the antigenicity of the antigen since metals including Zn have ability to modify structures of proteins[34,35]. In addition, the adjuvant effect of ZnO may be also explained by production of inflammatory cytokines such as IL-1 and TNF- α by ZnO-stimulated peritoneal macrophages since it was previously demonstrated that metallic particles increased the levels of such inflammatory cytokines from macrophages[36].

In summary, the present study demonstrates that ZnO appeared to have a marked adjuvant effect on immune responses, especially Th2 but not Th1 responses.

Acknowledgements

Declaration of interest: The authors declare no conflict of interest.

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