

Zinc oxide nanoparticles provide an adjuvant effect to ovalbumin via a T_H2 response in Balb/c mice

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Abstract

Zinc oxide nanoparticles (ZNPs) have been used in dietary supplements and may cause an immunomodulatory effect. The present study investigated the effect of ZNPs on antigen-specific immune responses in mice sensitized with the T-cell-dependent antigen ovalbumin (OVA). BALB/c mice were intraperitoneally administered ZNPs (0.25, 0.5, 1 and 3 mg) once, in combination with OVA, and the serum antibodies, splenocyte reactivity and activation of antigen-presenting cells were examined. The serum levels of OVA-specific IgG1 and IgE were found significantly enhanced by treatment with ZNPs over control. An increased level of IL-2, IL-4, IL-6, IL-17 and decreased level of IL-10 and TNF- α in splenocytes administered with ZNPs were observed in comparison with control. The ZNPs and OVA-stimulated T lymphocytes showed enhanced proliferation compared with control. Macrophages and B cells showed high expression of MHC class II, whereas higher expression of CD11b in macrophages of the ZNPs and ZNPs/OVA treated groups was observed. The lungs and spleen had increased eosinophils and mast cell numbers. Also, myeloperoxidase activity in lungs was found to be increased by 2.5-fold in the case of ZNPs and 3.75-fold increase in ZNPs/OVA, whereas in intestine, there was significant increase in both the groups. Increased expression of the genes for GATA-3, SOCS-3, TLR-4, IL-13 and IL-5 in the intestine was observed. Collectively, these data indicate that systemic exposure to a single administration of ZNPs could enhance subsequent antigen-specific immune reactions, including the serum production of antigen-specific antibodies, and the functionality of T cells.

Keywords: allergy, anaphylaxis, antigen-presenting cells, cytokines, mast cells

Introduction

Previous toxicological studies demonstrate that the LD₅₀ of ZnO in rats was 5 g kg⁻¹ and it was found non-toxic when given orally. Therefore, ZnO is categorized as a non-toxic chemical according to the globally harmonized classification system for the classification of chemicals (1). Zinc oxide nanoparticles (ZNPs) have been used as antibacterial, antifungal, dental filling material and UV-blocking agents in personal care products etc. (2–5). Despite the widespread use of ZNPs, several aspects of this compound for humans are still unclear. Single intra-peritoneal administration of ZNPs resulted in its accumulation in liver, spleen, lungs, kidney and heart (6). The immune organs have been reported as the main sites for the deposition of nanoparticles following systemic exposure (7, 8). Hence, the interaction between nanoparticles and

immune cells and thereafter the consequences of such interactions are relevant issues in addressing the potential health impact of ZnO and other nanoparticles. Therefore, a detailed investigation was required in terms of the safety of ZNPs.

Supporting a relationship between particles and increased allergic symptoms, particles have been shown to increase allergic responses both in humans and in animal models (9, 10). In rodent models, different particles [fly ash, carbon black, diesel exhaust particles, polystyrene particles, polytetrafluoroethylene (teflon), TiO₂ or amorphous silica particles] have been shown to increase allergen-specific IgE levels after inhalation, intra-tracheal, intra-nasal, intra-peritoneal and subcutaneous exposure (11–16). Inhalation exposure of ZnO microparticles (25 and 50 mg m⁻³) and instillation of

300nm ZNPs (1 and 5 mg kg⁻¹ body weight) of rats resulted in transient inflammation measured in the bronchoalveolar lavage (BAL) by showing increased lactate dehydrogenase (LDH) release, protein and neutrophil content (17). Single intra-tracheal instillation of rats with smaller particles of 50–70nm ZNPs and <1000nm ZnO microparticles (1 and 5 mg kg⁻¹ body weight) resulted in potent but reversible inflammation (measured in the BAL by increased LDH release, cell number and neutrophil content) (18). Furthermore, single intra-tracheal instillation of rats with <10nm ZNPs induced an eosinophilic/fibrotic/granulomatous inflammation (19) and recruitment of eosinophils and neutrophils in the BAL (20).

Particulate matter, such as pathogens and also nanoparticles, is readily ingested by monocytes/macrophages and dendritic cells. This may pose a hazard for these cell types, thereby reducing the capacity to mount an appropriate immune response. Reports of ZNPs affecting macrophage functions are available, such as expression of IL-1 β and chemokine CXCL9 induced by 20nm ZNPs in murine bone-marrow-derived dendritic cells (BMMCs) and RAW264.7 murine macrophages (21). IFN- γ , TNF- α and IL-12 production got induced by ZNP exposure to PBMCs (22).

Collectively, these reports demonstrated that exposure to ZNPs affects macrophage and T cell mediated immune reactions. However, it is currently unclear whether antigen-specific immunity is influenced by ZNPs or not. In light of the available evidence showing the impacts of ZNPs on the functionality of macrophages and T cells, the objective of the present study was to investigate the effect of ZNPs on antigen-specific immune responses in Balb/c mice sensitized with ovalbumin (OVA), a T-cell-dependent antigen.

Materials

Animals

Inbred strains of female Balb/c mice (8–10 weeks old, 18–20g) procured from the animal breeding colony of CSIR-Indian Institute of Toxicology Research (Lucknow, India) were acclimatized under standard laboratory conditions for 1 week prior to the experiment. Animals were housed in polycarbonate cages and maintained at room temperature under standard laboratory conditions of light and dark cycles (12–12h). Animals were kept on normal diet and water *ad libitum*. This study was carried out after the approval of Institutional Ethics Committee (approval no. IITR/IAEC/47/11).

Endotoxin detection

The Limulus amoebocyte lysate (LAL) assay was performed to detect endotoxin contamination in OVA and ZNPs with the Pierce LAL Chromogenic Endotoxin Quantification kit (PI-88282, U. BEE. Scientific, USA) according to the manufacturers' protocol.

Animal experiment protocol

The animal study was carried out according to the method described earlier (23). In brief, mice were randomly divided into the four groups (5 animals per group): naive (NA), endotoxin-free OVA-sensitized, endotoxin-free ZNPs alone (<50nm, Sigma) and the combination of ZNPs and OVA

sensitization (ZNPs/OVA). Varying doses (1, 2, 4 and 12mg ml⁻¹) of nanoparticles were suspended in PBS, pH 7.4. The nanoparticle suspension and the antigen (OVA; 400 μ g ml⁻¹) solution were mixed together in equal volumes using a vortex mixer for few seconds (20–30 s). Mice were then immediately immunized by intra-peritoneal injections of 500 μ l containing varying doses (0.25, 0.5, 1 and 3mg) of nanoparticles and/or OVA (100 μ g) on day 0. Two booster doses of OVA (1 μ g ml⁻¹) were given to each mouse on the 21st and 23rd day and they were sacrificed on the 30th day after challenge with 1 mg ml⁻¹ of OVA. Blood samples from individual mice were collected on both days, and the serum was obtained by centrifugation of the coagulated blood at 3000 $\times$ g for 15 min.

Systemic anaphylaxis score and rectal temperature

Anaphylactic reactions were scored using a previously described method (24): 0—no symptoms; 1—scratching and rubbing around the snout and head; 2—puffiness around the eyes and snout, pilar erection, diarrhea and reduced activity or standing still with an increased respiratory rate; 3—wheezing, labored respiration and cyanosis around the mouth; 4—symptoms as mentioned in score 3 with loss of consciousness, tremors and/or convulsion; 5—death.

The rectal temperature was monitored after 20 min of challenge using a digital rectal thermometer (Bioseb, France).

Immunohistochemical (IHC) analysis of eosinophil and mast cell staining

Detection of eosinophils in the lungs and spleen was performed using IHC methods as described earlier (25). Mast cell staining in the tissue sections of the lungs, spleen and intestine was carried out using Toluidine blue stain and the images were taken using a microscope (Nikon Eclipse TE2000-S microscope).

Histopathology

Spleen, lungs and intestines of challenged mice were taken for histopathology. Tissues were fixed in 10% formalin in PBS, embedded in paraffin and cut into 3–5 μ m thick sections. The images were captured by a microscope (Leica microsystem, Germany).

Measurement of OVA-specific IgG and IgE

OVA-specific IgG1 and IgE were measured as previously described (26). In brief, ELISA plates were coated with 0.05% OVA in coating buffer (0.1M NaHCO₃, pH 7) and blocked with 3% BSA containing 0.05% Tween 20 (PBS-T). After washing with PBS-T, serum samples from all groups were placed into wells (1:10 v/v) and incubated overnight at 4°C. HRP-conjugated anti-mouse IgE (1:1000 v/v; Sigma Chemical Co., St Louis, MO, USA) was used as a secondary antibody. Lastly, wells were washed and a tetramethylbenzidine substrate solution (BD Biosciences, San Jose, CA, USA; 50 μ l per well) was added for colorimetric detection of bound peroxidase conjugate. The reaction was stopped by adding 50 μ l per well of 5N H₂SO₄. The absorbance was measured at 450 nm using an ELISA plate reader (Biotek, Power Wave XS2, USA).

Splenocyte culture and cytokine measurement

Spleens from the different groups were taken and single cell suspensions were prepared aseptically in RPMI (Sigma, St Louis, MO, USA) according to a previously described method (27). The splenocytes (2×10^6 cells ml^{-1}) were cultured in 96-well plates (100 μl per well) and stimulated with OVA (50 $\mu\text{g ml}^{-1}$) for 72h. The supernatants were collected and quantified for RANTES, IL-2, IL-4, IL-6, IL-17, IL-10, IFN- γ and TNF- α by using a cytometric bead array flow cytometry kit and Flex set (BD Biosciences, San Jose, CA, USA).

Monocyte chemotactic protein-1 (MCP-1) in serum

The MCP-1 level in serum was measured by a commercially available ELISA kit (BD Biosciences, Germany) according to the manufacturers' protocol in treated and control groups.

Lymphoproliferation assays

The lymphoproliferation assay was carried out according to the method described earlier (28). ConA was used to induce proliferation in T-cell populations present in splenocyte culture. Cultured splenocytes were treated with ConA (5 $\mu\text{g ml}^{-1}$) in the presence of OVA (50 $\mu\text{g ml}^{-1}$) and incubated at 37°C for 72h. The proliferation rate of cells was determined by the uptake of tritiated thymidine by cells. Tritiated thymidine (2 $\mu\text{Ci ml}^{-1}$) was added to the cultures 18h prior to the harvesting of cells. The cells were harvested with Nunc cell harvester on glass fiber filters. These filters were transferred to scintillation cocktail-W (Sisco Research Laboratories Pvt. Ltd Mumbai, India), and β -counts were recorded on a β -counter (Hewlett-Packard, Palo Alto, CA, USA).

Lymphocyte populations and their functionality

Single cell suspensions of splenocytes were stained with fluorochrome-conjugated antibodies. For measuring activated B cells, splenocytes were suspended in staining buffer (2% FBS, 1% sodium azide in PBS) and stained for 20min on ice with Alexa fluor 700-conjugated anti-CD19 for marking B cells and FITC-conjugated anti-MHC class II. For T-lymphocyte phenotyping, cells were stained with FITC-conjugated anti-CD4 for T_H cells, allophycocyanin-conjugated anti-CD8 for T_C cells and allophycocyanin-Cy7-conjugated anti-CD3e. The stained cells were washed

twice with wash buffer (0.01% sodium azide in PBS) and finally suspended in 500 μl PBS. After washing, cells were acquired by using a flow cytometer (FACS Canto II, BD BioSciences, San Jose, CA, USA).

Antigen-presenting markers of macrophages

Cell-surface markers were studied on peritoneal macrophages. Peritoneal exudate cells were collected from the peritoneal cavity of from each group ($n = 5$) by injecting chilled RPMI 1640 medium and 2×10^6 cells per well was added to 12-well cell culture flat bottom plates. After 3h of incubation in a CO_2 incubator (5% CO_2) at 37°C, the non-adherent cells were removed by vigorous washing (three times) with warm RPMI 1640 medium. Furthermore, adhered cells were stained with PE-conjugated anti-class I MHC, FITC-conjugated anti-class II MHC and Alexa fluor 488-conjugated anti-CD11b for 30min at 4°C. After 30min of incubation, cells were washed with PBS and analyzed on a flow cytometer.

Myeloperoxidase (MPO) activity

MPO activity was determined by the method of Cardoso *et al.* (29). In brief, different samples were weighed (100mg) and homogenized in 3ml of 0.5% hexadecyl trimethyl ammonium bromide in 50mM phosphate buffer, pH 6.0, by using a homogenizer on ice. After three freeze thaw cycles, homogenates were centrifuged at $40\,000 \times g$ for 15min, and the MPO activity of the supernatant was measured by incubating 20ml of diluted sample with 180ml of 50mM potassium phosphate buffer, pH 6.0, containing 0.157mg ml^{-1} *o*-dianisidine dihydrochloride (Sigma, USA) and 0.0005% of H_2O_2 . Absorbance was measured at 450nm using an ELISA plate reader (Biotek, USA). The MPO activity was expressed as units per weight (milligram) of tissue.

Semi-quantitative RT-PCR

A semi-quantitative RT-PCR analysis of T_H2 cytokines (IL-4, IL-5 and IL-13, IL-10 genes) in the intestine of mice and transcription factors [GATA-3, suppressor of cytokine signaling 3 (SOCS-3), T-bet and Foxp3 genes] and the TLR-4 gene in the intestine was carried out using gene-specific primers in control, ZNPs, OVA and ZNPs/OVA groups. The oligonucleotide primers used are listed in Table 1. Total

Table 1. Primers sequences used for PCR

Gene	Forward primer	Reverse primer
IL-4	5'-TCGGCATTGTAACGAGGTC-3'	5'-AAAAGCCCCGAAAGAGTCTC-3'
IL-5	5'-TCACCGA GCTCTGTTGACAA-3'	5'-CCACACTTCTCTTTTGGCG-3'
IL-13	5'-GACCCAGAGGATATTGCATG-3'	5'-CCAGCAAAGTCTGATGTGAG-3'
IL-10	5'-TGCCTGCTCTACTGACTGG-3'	5'-CTGGGAAGTGGGTGCAGTTA-3'
β -actin	5'-GATTACTGCTCTGGCTCCTAG C-3'	5'-GACTCAT GTACTCCTGCTTGC-3'
GATA-3	5'-TCTCACTCTCGAGGCAGCATGA-3'	5'-GGTACCATCTCGCCGCCACAG-3'
T-bet	5'-TCCCATTCTCTGCTTCA-3'	5'-GCTGCCTTCTGCCTTTC-3'
SOCS-3	5'-GCGAGAAGATTCCGCTGGTA-3'	5'-AAACCTGGGCTCCAAGATGG-3'
Foxp3	5'-GCTCCCGGCCTG GTCTGCTC-3'	5'-AGGTGGCGGGGTGGTTTCTGA-3'
TLR-4	5'-GCTCCCGGCCTGGTCTGCTC-3'	5'-AGGTGGCGGGG TGGTTTCTGA-3'
GAPDH	5'-TTCACCACCATGGAGAAGGC-3'	5'-GGCATGGACTGT GGTCTGA-3'

β -actin and GAPDH are internal controls.

RNA from intestine was isolated with RNAzol®RT (Molecular Research Center, Inc. Cincinnati, OH, USA). cDNA from different groups was prepared by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. The RT-PCR was carried out using a PCR master mix (Thermo Scientific, Waltham, MA, USA) according to the manufacturers' instructions. After completion of PCR cycles, 20 µl of PCR products were analyzed in 2% agarose gel electrophoresis. The density of each band was estimated by the Genetools software (Syngene). The β -actin and GAPDH were taken as endogenous controls and their respective densitometry values were used for normalizing different mRNA expression in the intestines. Normalized values were used for plotting the bar graphs.

Statistical analyses

The statistical significance of the data obtained was determined using a software package from InStat version 5.0 (Graph pad, San Diego, CA, USA; <http://www.graphpad.com>) using the Bonferroni analysis of variance test. Values for all measurements have been expressed as mean $\pm$ SEM. Differences between groups were considered significant at $P < 0.05$.

Results

Effect of ZNPs on anti-OVA IgG1 and IgE production

Since the levels of specific IgG1 and IgE were found enhanced at the dose of 0.25mg of ZNPs/OVA, therefore all further studies were carried out on the aforesaid dose. To measure humoral responses, serum sample from individual mice was collected on the 21st and 23rd day after the OVA sensitization and OVA-specific antibodies were examined. A marked increase in the levels of OVA-specific IgG1 and IgE were observed in ZNPs/OVA mice group compared with naive and OVA-treated mice (Fig. 1a and b). The production of OVA-specific IgG1 was attenuated by doses from 0.5 to 3mg (data not shown), whereas at the 0.25mg dose, it was significantly enhanced. Similarly formation of OVA-specific IgE was significantly increased at 0.25mg ZNPs/OVA. ZNPs alone failed to exert any effect.

Anaphylaxis score and rectal temperature

Twenty percent of the animals in the ZNPs-alone group exhibited scratching and rubbing around the snout and head (score 1); 20% of the animals showed puffiness around the eyes and snout, pilar erection, diarrhea and reduced activity or standing still with an increased respiratory rate (score 2); 60% of the animals exhibited

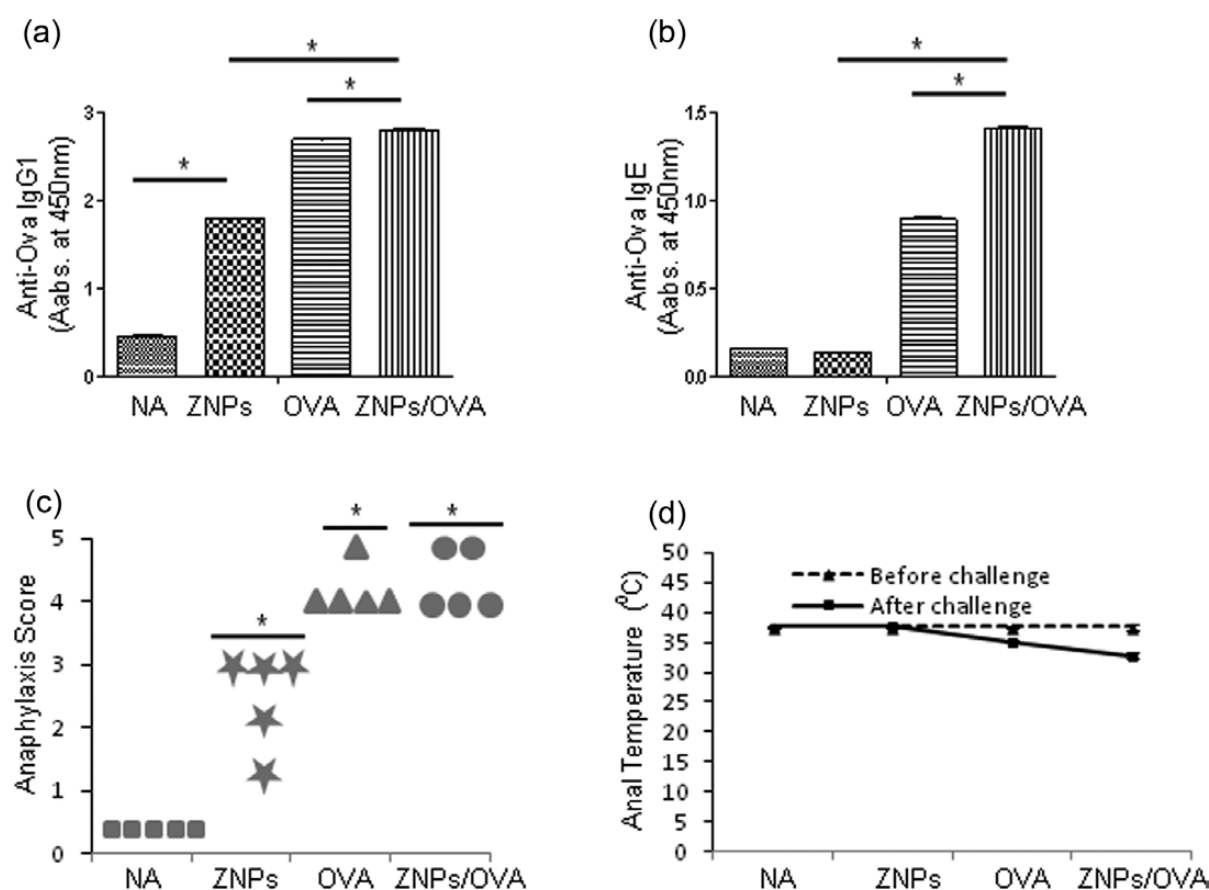


Fig. 1. Specific immunoglobulin levels and anaphylactic symptoms. (a) anti-OVA IgG1 and (b) anti-OVA IgE levels, (c) anaphylaxis score and (d) rectal temperatures in the control (NA), ZNPs, OVA and OVA/ZNPs groups. Data were expressed as means $\pm$ SEM of five replicates (different symbols indicate different groups and their number indicate number of animals in a particular group). Values represented are significantly different from NA versus the other groups, ZNPs versus ZNPs/OVA and/or OVA versus ZNPs/OVA at $*P < 0.05$.

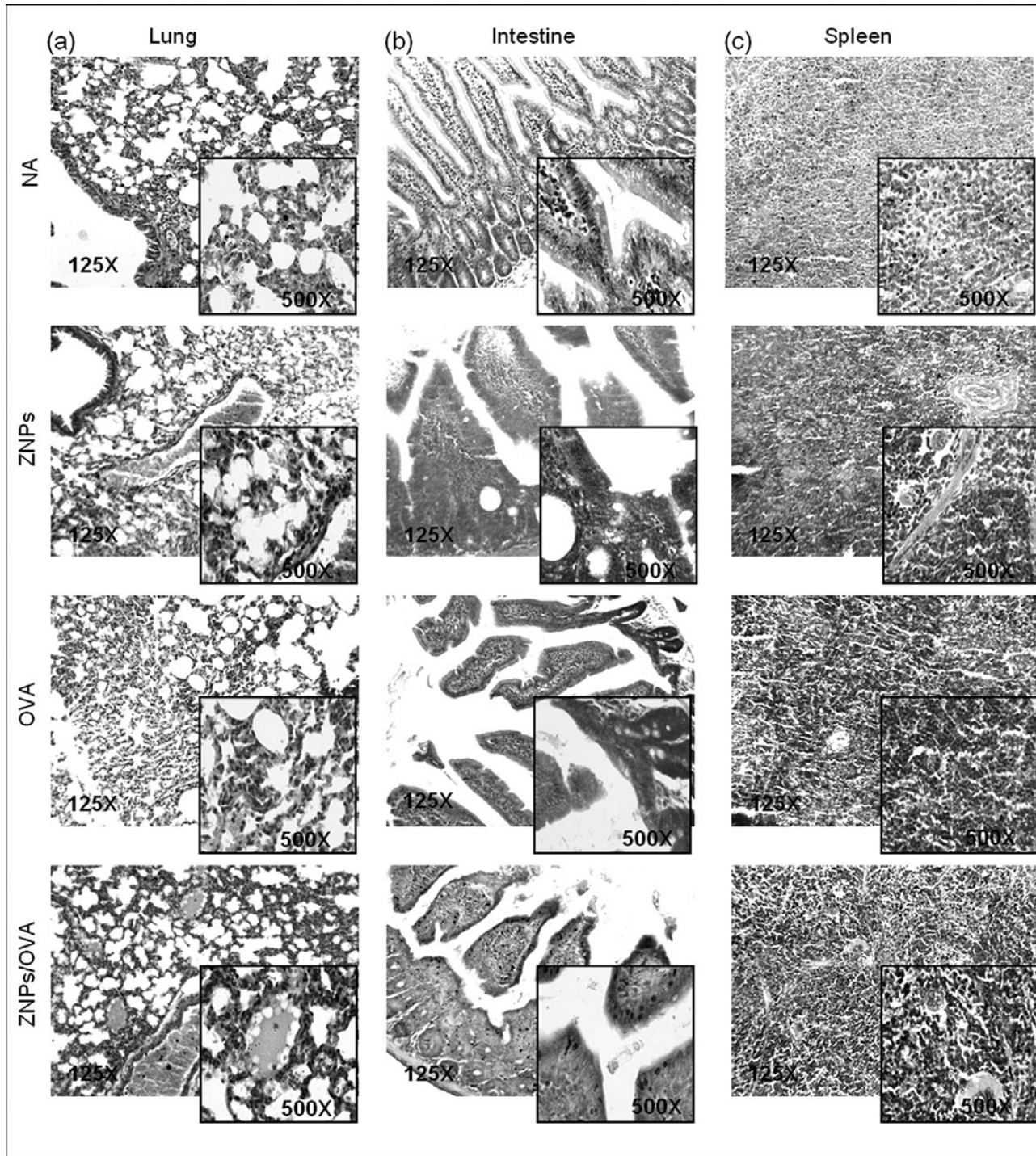


Fig. 2. Histopathological changes in different organs. (a) Lungs, (b) intestine and (c) spleen of the control (NA), ZNPs, OVA and OVA/ZNPs groups.

wheezing, labored respiration and cyanosis around the mouth (score 3). Eighty percent of animals from the OVA group showed wheezing, labored respiration and cyanosis around the mouth, loss of consciousness, tremors and/or convulsion (score 4) and 20% of the animals died (score 5). Further, 60% of OVA/ZNPs-group animals demonstrated similar anaphylactic symptoms to

those of the OVA group (score 4) with increased mortality (score 5) in 40% of the animals. Naive mice showed no reaction to challenge (Fig. 1c). Temperatures in the naive group ranged between 37 and 38°C after OVA challenge, whereas rectal temperatures in 8 out of 10 mice in the ZNPs/OVA-treated group were 4.9°C below the normal range of the control group (Fig. 1d).

Mast cells

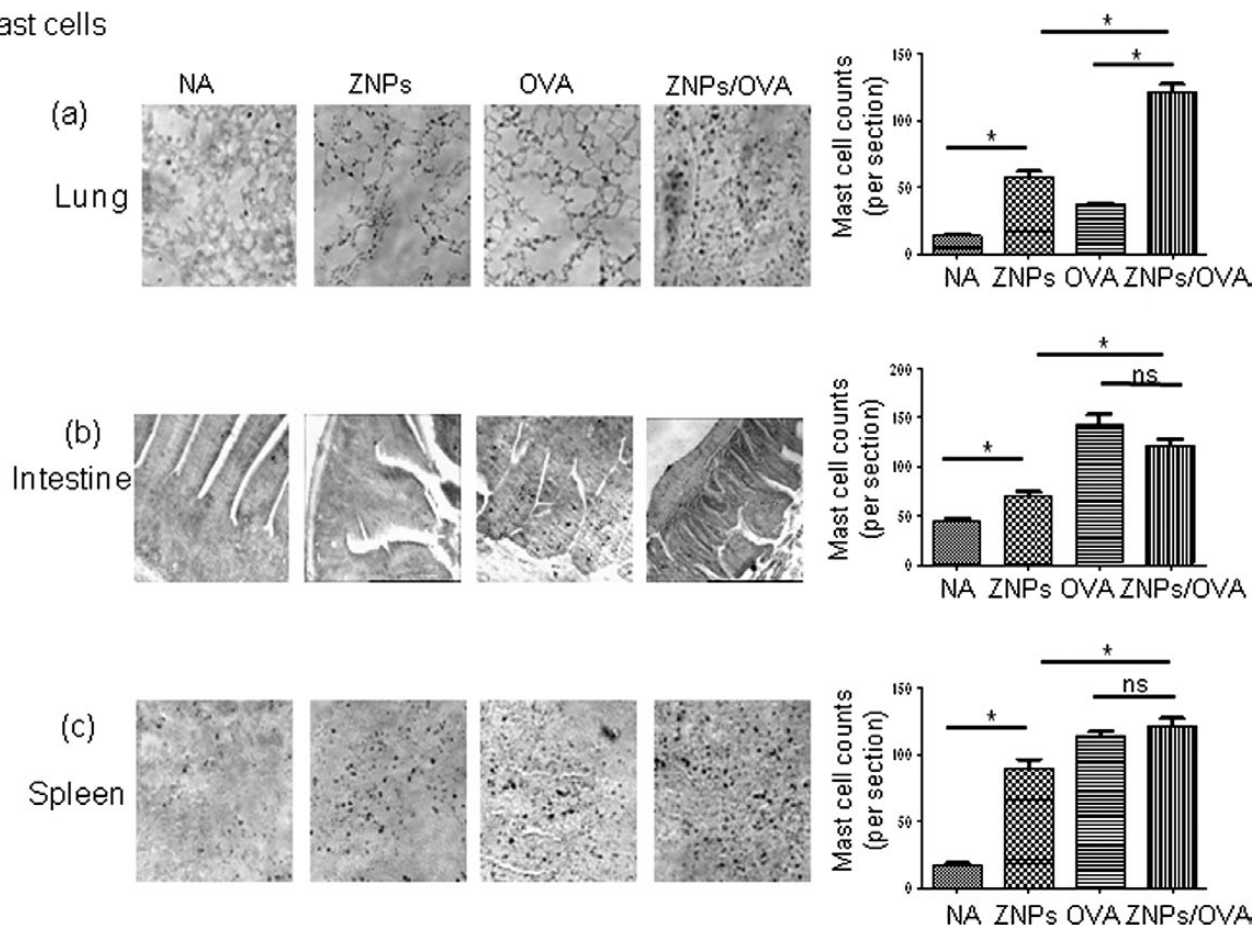


Fig. 3. Mast cell levels in the organs. (a) Mast cells in the lungs, (b) intestine and (c) spleen of the control (NA), ZNPs, OVA and OVA/ZNPs groups. Data are expressed as means $\pm$ SEM of five replicates. Values represented are significantly different in NA versus ZNPs, ZNPs versus ZNPs/OVA and/or OVA versus ZNPs/OVA at $*P < 0.05$ and ns = non-significant.

Histopathology

In ZNPs/OVA-treated mice, mixed inflammatory cell infiltration throughout the lung parenchyma was observed, whereas edema to some extent and moderate congestion were also found (Fig. 2a). Prominent exfoliations of mucosa in the lumen, infiltration by inflammatory cells, mucosal gland hypertrophy and denudation of mucosal tips were observed in the intestine (Fig. 2b). The spleen was observed to have severe megakaryocytic hyperplasia and hypertrophy (Fig. 2c). In the OVA-treated group, lungs showed edema at few places and moderate infiltration by mixed inflammatory cells. The spleen exhibited hyperplasia, whereas the intestine showed hyperplasia of enterocytes and exfoliation of the mucosa in the lumen. The ZNPs-alone-treated group was found to have congestion and thickening of alveolar septa, while the spleens in this group showed lymphoid hyperplasia and the intestine had hypertrophied goblet cells and exfoliation of mucosal tips. The control group showed normal histology of lungs, intestine and spleen.

Mast cell and eosinophil counts in different organs

A marked increase in the number of mast cells in the intestine, lungs and spleen in ZNPs and ZNPs/OVA mice in comparison with control and OVA-treated mice was observed (Fig. 3a–c).

The number of eosinophils was enhanced in the lungs and spleen, whereas in the intestine, no change was observed (Fig. 4a–c).

MPO activity in the lungs and spleen

Total MPO activity per unit weight of the lungs and intestine was found significantly elevated in ZNPs and ZNPs/OVA group with respect to control and OVA-treated mice (Fig. 4D and E).

Activation markers on macrophages

All tested groups showed a significant increase in MHC I in comparison with controls and in the case of class II MHC expression, the OVA and ZNPs/OVA-treated groups had significantly increased expression (Fig. 5A and B). The ZNPs/OVA group had significant increased expression of CD11b over macrophages in comparison with control macrophages (Fig. 6).

Effects of ZNPs on lymphocyte populations and functionality

The cellularity of splenocytes was examined by flow cytometry, and a significant difference was observed in the percentage of

Eosinophils

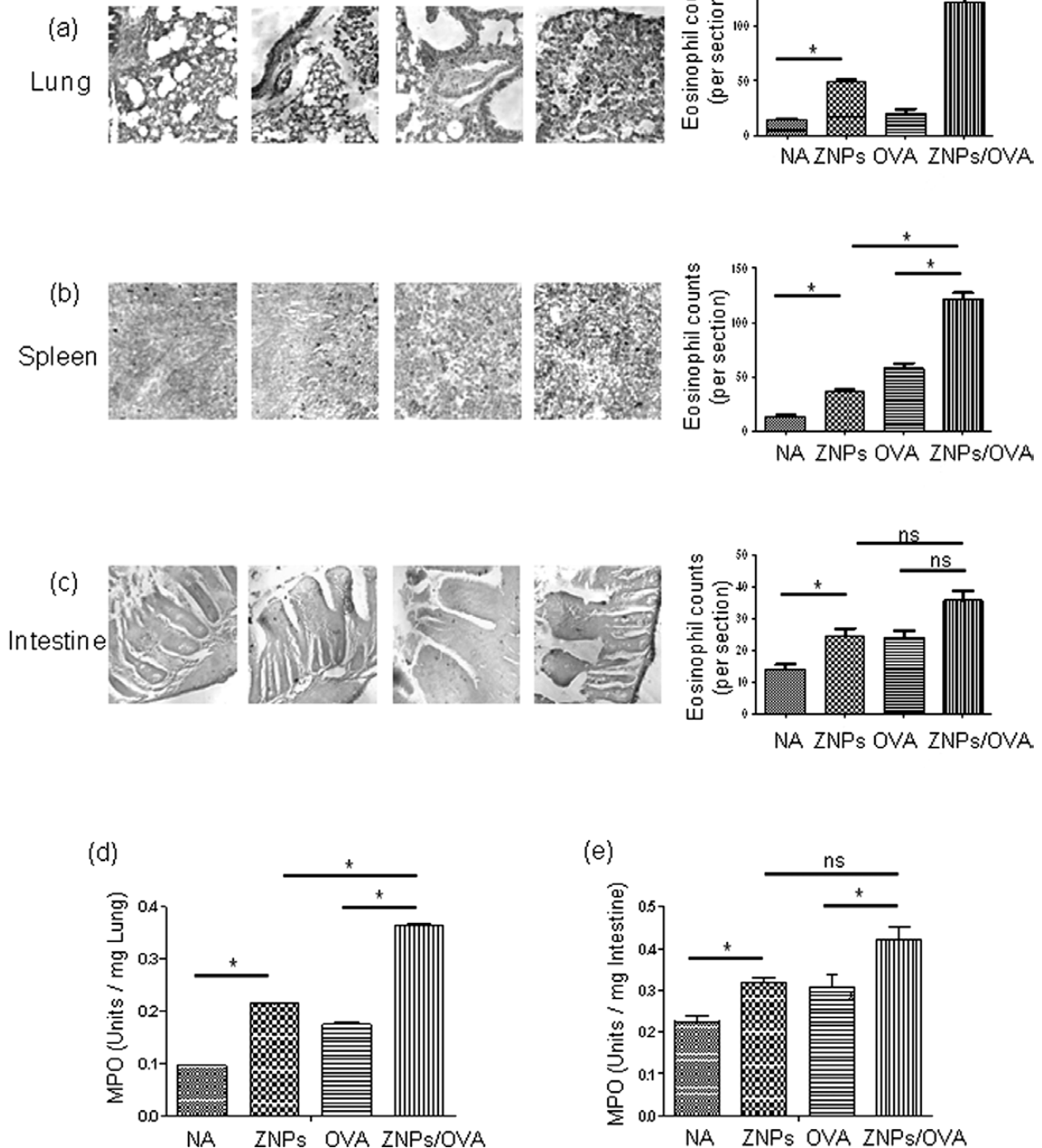
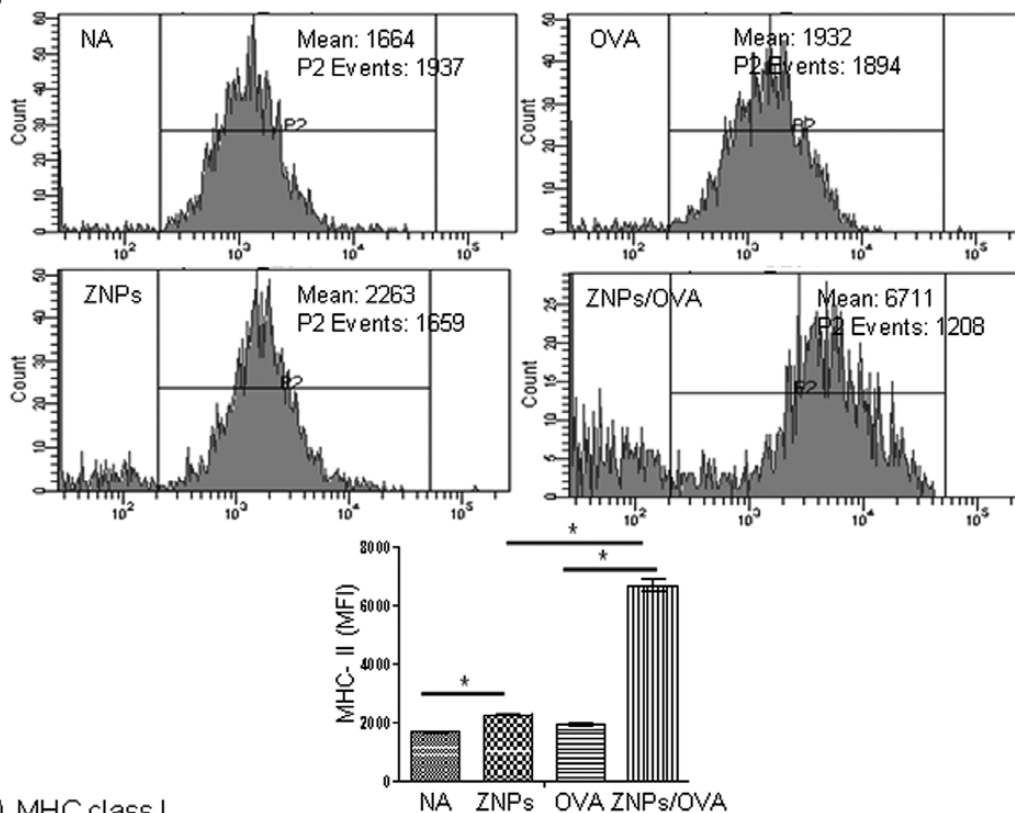


Fig. 4. Eosinophils and MPO in the organs. (A) Eosinophils in the lungs, (B) intestine and (C) spleen. (D) Total MPO activity per unit weight of the lungs and (E) intestine of the control (NA), ZNPs, OVA and OVA/ZNPs groups. Data are expressed as means $\pm$ SEM of five replicates. Values represented are significantly different in NA versus ZNPs, ZNPs versus ZNPs/OVA and/or OVA versus ZNPs/OVA at $*P < 0.05$ and ns = non-significant.

splenic CD4⁺, CD8⁺ and MHC-II⁺ CD19⁺ cells among control and nanoparticle-treated groups (Fig. 7A–C). There was significant increase in CD4⁺ cells in all the tested groups (range from 48 to 53%) compared with control (38%), whereas in the case of CD8⁺ cells, only ZNPs-treated and ZNPs/OVA-treated

groups had shown significant increase from 14.7% (CD8⁺ in controls) to ~20% (CD8⁺ in the ZNPs and ZNPs/OVA-treated groups). The percentages of activated B cells (MHC-II⁺ CD19⁺) were elevated by 26% in ZNPs and 28% in ZNPs/OVA-treated groups (Fig. 7B).

(a) MHC class II



(b) MHC class I

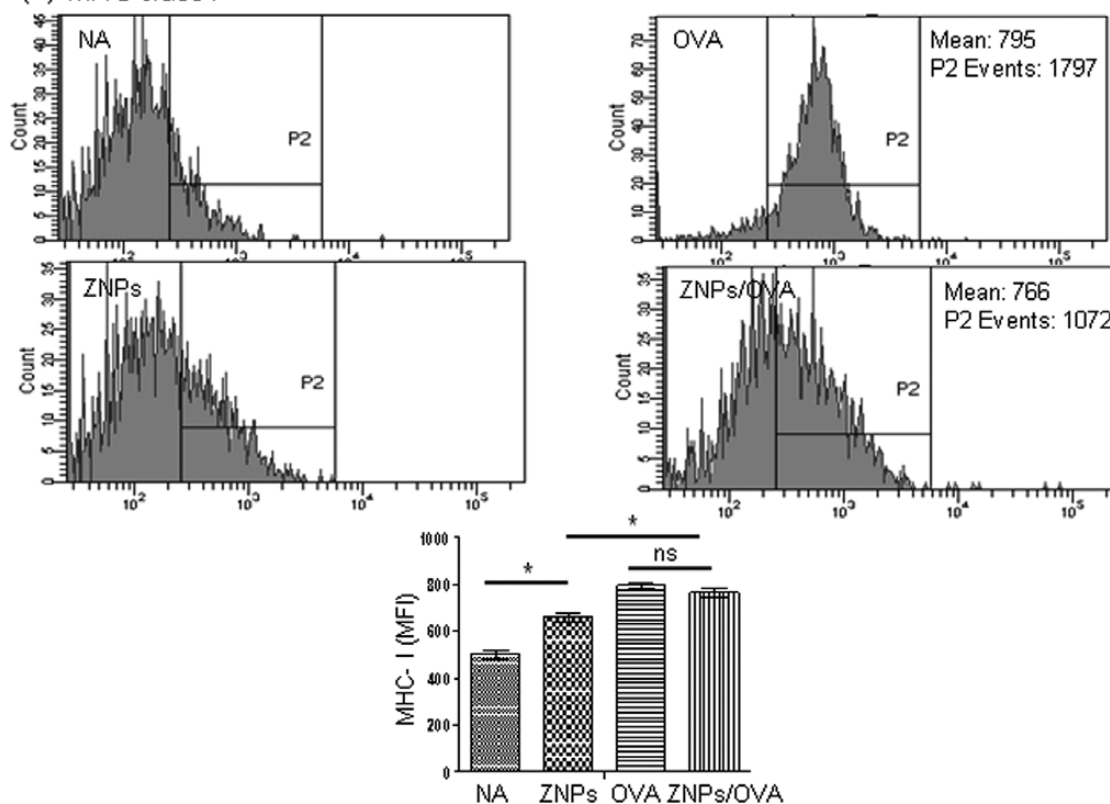


Fig. 5. Major histocompatibility complexes (I and II) expression. (A) MHC class II and (B) MHC class I markers on macrophages of the control (NA), ZNPs, OVA and OVA/ZNPs groups. Data are expressed as means $\pm$ SEM of three replicates. Values represented are significantly different in NA versus ZNPs, ZNPs versus ZNPs/OVA and/or OVA versus ZNPs/OVA at $*P < 0.05$ and ns = non-significant.

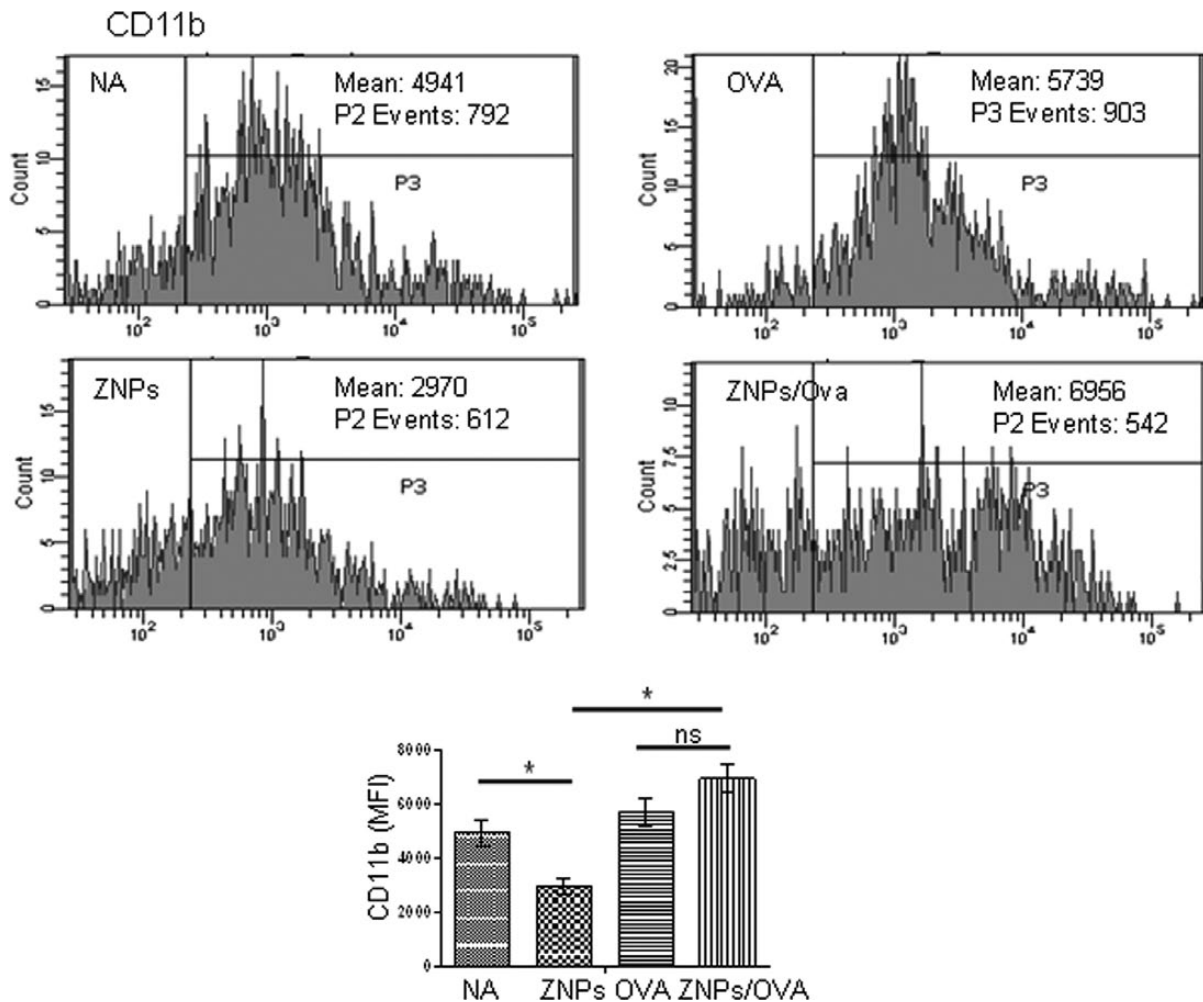


Fig. 6. CD11b expression. CD11b activation markers on macrophages of the control (NA), ZNPs, OVA and OVA/ZNPs groups. Data are expressed as means $\pm$ SEM of three replicates. Values represented are significantly different in NA versus ZNPs and ZNPs versus ZNPs/OVA at $*P < 0.05$ and ns = non-significant.

Effect of ZNPs on ConA-stimulated lymphoproliferation in splenocytes

Mitogen-driven lymphoproliferation has been used as a test to study immunomodulation by biological response modifier agents; therefore, to monitor the influence of ZNPs on lymphocyte proliferation, splenocytes were stimulated with ConA, a T-cell mitogen, for 72h and the proliferation was measured. Compared with the ConA-stimulated lymphocytes, a significant increase in proliferation was found in the OVA and ZNPs groups only (Fig. 7C).

GATA-3, SOCS-3, Foxp3, T-bet, TLR-4 and T_H2 cytokine mRNA expression in the intestine

We further extended our study to evaluate the effect of ZNPs in the intestine. The mRNA levels of GATA-3, TLR-4, SOCS-3, IL-4, IL-5 and IL-13 were found to be significantly ($P < 0.05$) elevated in the ZNPs/OVA group compared with other groups. Further, the level of T-bet was found to be reduced and no change was observed in Foxp3 (Fig. 8).

Effect of ZNPs on secretion of cytokines and MCP-1

As T cells play a pivotal role in antigen-specific humoral responses, we examined the effect of ZNPs on the functionality of T cells. The amount of cytokines produced by splenocytes of the non-sensitized mice was very low, and the OVA stimulation showed markedly increased production of IL-2, IL-4, IL-6 and IL-17 and decreased levels of IL-10 and TNF- α . For IFN- γ , no significant change was observed between ZNPs and ZNPs/OVA groups (Fig. 9). Further, the levels of MCP-1 were found elevated in the serum of ZNPs, OVA and ZNPs/OVA groups, but the relative significance of MCP-1 was higher in the ZNPs/OVA group (Fig. 10).

Discussion

In this study, we have demonstrated for the first time that even a brief ZNP exposure is sufficient to boost the secondary immune response of prior OVA-sensitized animals. We have taken the doses from 0.25 to 3mg and observed that the lower dose was highly effective for the generation of humoral responses. A better adjuvant should have the potential of

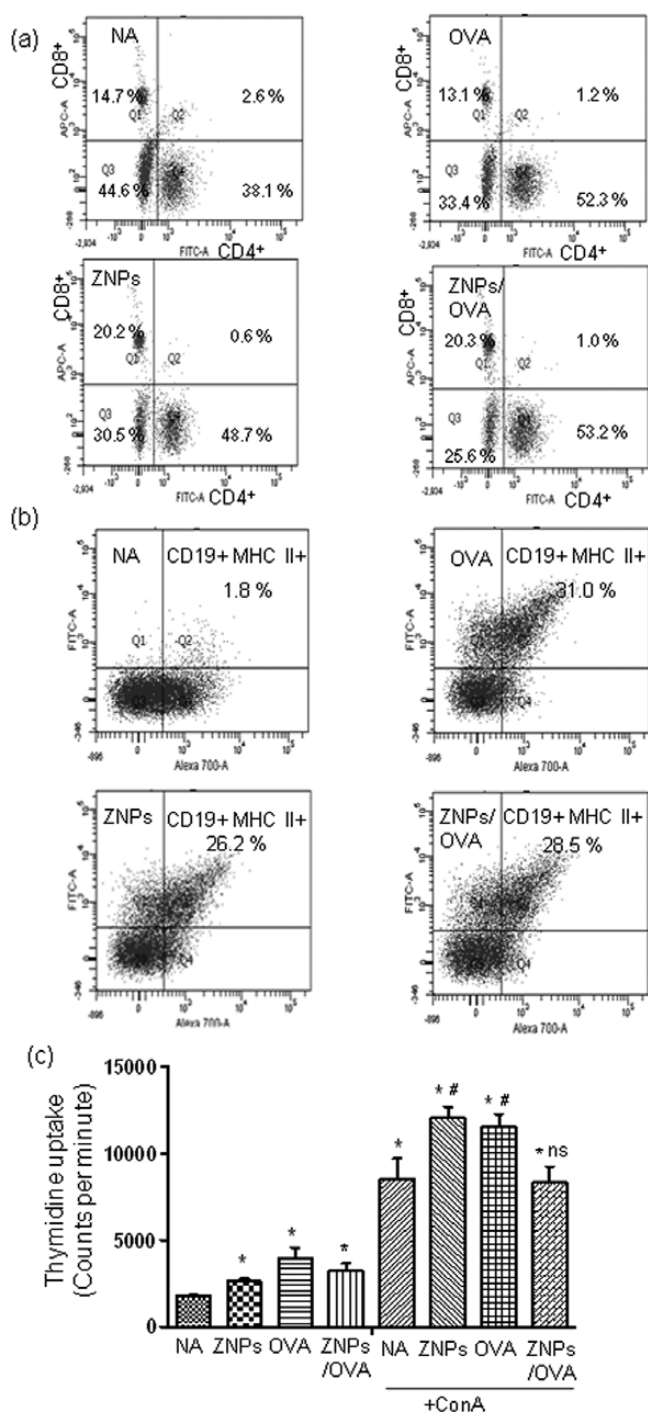


Fig. 7. Lymphocyte populations and lymphoproliferation. (A) CD4 and CD8 cells (B) activated B cells or CD19⁺MHC-II⁺ and (C) lymphoproliferation on ConA stimulation in *ex vivo* splenocytes of control (NA), ZnPs, OVA and OVA/ZnPs groups. Data are expressed as means $\pm$ SEM of three replicates. Values represented are significantly different from NA at * $P < 0.05$; # $P < 0.05$ in the presence of conA and ns = non-significant.

enhancing antigen presentation and other humoral responses of APCs at lower doses so that it could be safer and not increase the particle load in the immune system (26, 27). Our study showed that a single intra-peritoneal administration

of ZnPs significantly enhanced the subsequent production of antigen-specific antibodies, as evidenced by a marked increase in the serum levels of OVA-specific IgE and IgG1. The increase in these antibodies suggested that ZnPs appear to have an adjuvant effect toward a T_H2 response. Further, evidence for the efficacy of ZnPs as an adjuvant can be gleaned from the quality and levels of other responses elicited.

Cytokines play a critical role in the activation and differentiation of B cells. In particular, the class switching from IgM to IgG1 and IgE is dictated by T_H2 (i.e. IL-4) cytokines (28). In line with the enhancement of IgG1, several cytokines such as IL-2, IL-4, IL-5, IL-6 and IL-13 were found enhanced and TNF- α and IL-10 were decreased in response to ZNP exposure with allergen, suggesting that the increased antibody production may be attributed to the changes in T_H cell functions. Antigen-primed CD8⁺ T cells are required for the full development of airway hyper-response and airway inflammation, and this appears to be associated with the production of IL-13 from these primed T cells (29).

Antibody production is a complex process that requires interactions among various immune cells and the action of many mediators. Stimulation or increase in the percentage of T_H clones (CD4⁺ lymphocytes) increases the activation and production of cytokines. Lymphoproliferation in ZnPs, OVA and ZnPs/OVA groups was found compared with control, and on ConA stimulation, the OVA and ZnPs groups showed an increase in proliferation. Although, in the case of the ZnPs/OVA group, not much increased proliferation was found but still it was able to activate B cells (CD19⁺ MHC-II⁺ cells) by inducing changes in the expression of surface molecules and cytokine synthesis. Collectively, these results indicate that systemic exposure to a single dose of ZnPs enhanced antigen-specific T-cell responses. These findings reveal that allergen-specific CD4⁺ and CD8⁺ lymphocytes may have a role in the induction of eosinophilic airway inflammation. ZNP exposure significantly boosts eosinophilic inflammation in the lungs and spleen. The potency of ZnPs in terms of their pro-inflammatory and pro-allergic effects reside in their unique physical and chemical properties, including their small size and large surface area. Since OVA is a T-cell-dependent antigen that requires to be processed and presented by APCs (30), we postulated that APCs may also be involved in ZNP-mediated production of antibody production. Thus, in addition to T cells, other targets or mediators may be affected by ZnPs and contribute to the impaired humoral immunity.

T_H2 cells and their cytokine products such as IL-4 and IL-5 are associated with the induction of immediate-type hypersensitivity reactions such as asthma (31, 32). Eosinophils are common cells found to be linked with asthma and allergy (33). In our study, the increased levels of eosinophils in the ZnPs/OVA group indicate the severity induced by ZnPs in the presence of OVA.

Mast cells are among other cells responsible for the production of various allergic mediators such as IL-1 β , IL-5, CCL2 and RANTES (34, 35). IL-5 and RANTES attract eosinophils to the site of injection; however, mast cells, macrophages, neutrophils and eosinophils are dispensable for the adaptive immune response (36). Neutrophils are a principal component of the innate immune system and provide a first line of defense. Neutrophils can recruit inflammatory CD11b⁺

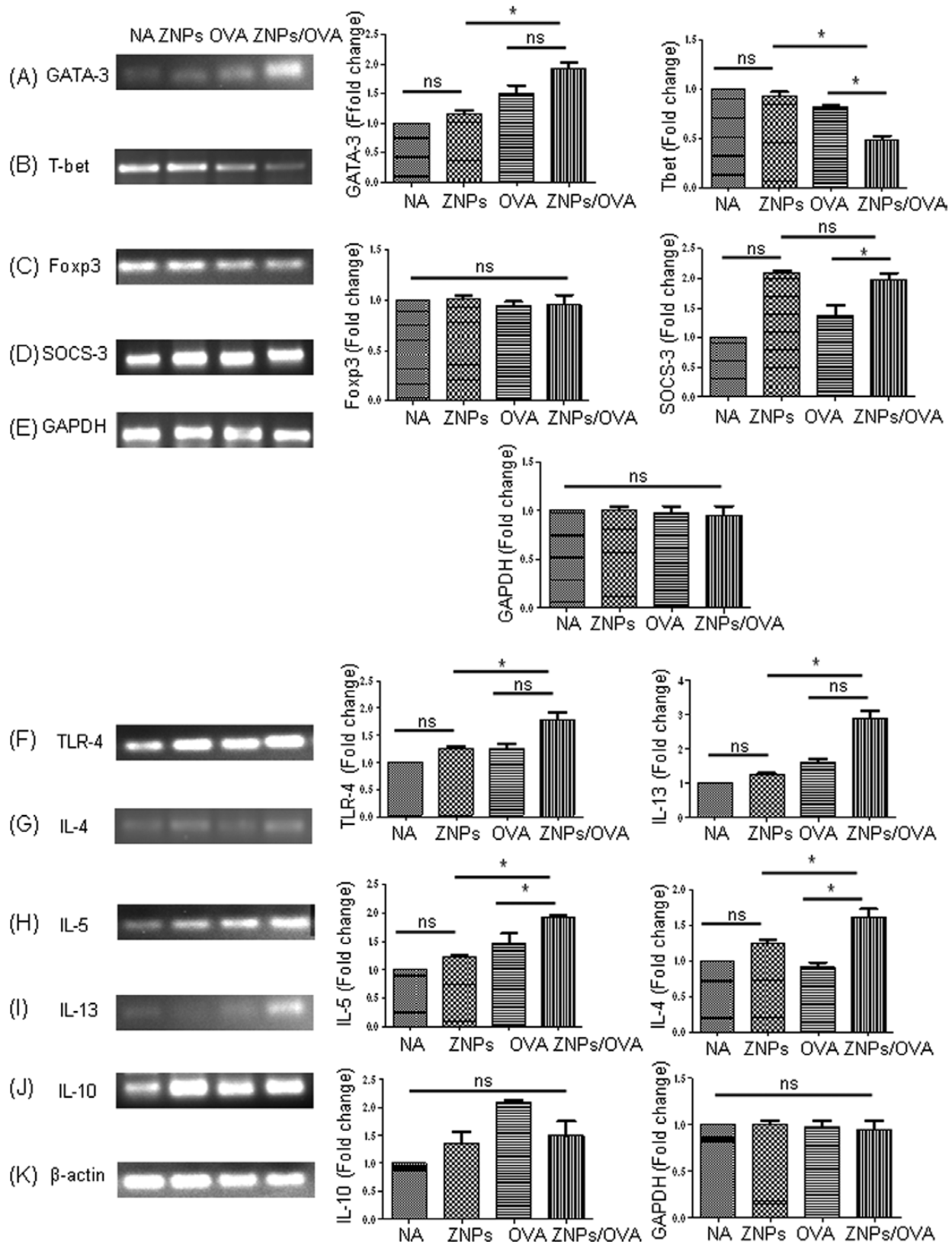


Fig. 8. The mRNA expressions of transcription factors and cytokines in the intestine (A) GATA-3, (B) T-bet, (C) Foxp3, (D) SOCS-3, (E) GAPDH, (F) TLR-4, (G–J) T_H2 cytokines, IL-4, IL-5, IL-13 and IL-10 and (K) β -actin of the control (NA), ZNPs, OVA and OVA/ZNPs groups. For densitometry, data are expressed as means $\pm$ SEM of three replicates. Values represented are significantly different in ZNPs versus ZNPs/OVA and/or OVA versus ZNPs/OVA at $*P < 0.05$ and ns = non-significant.

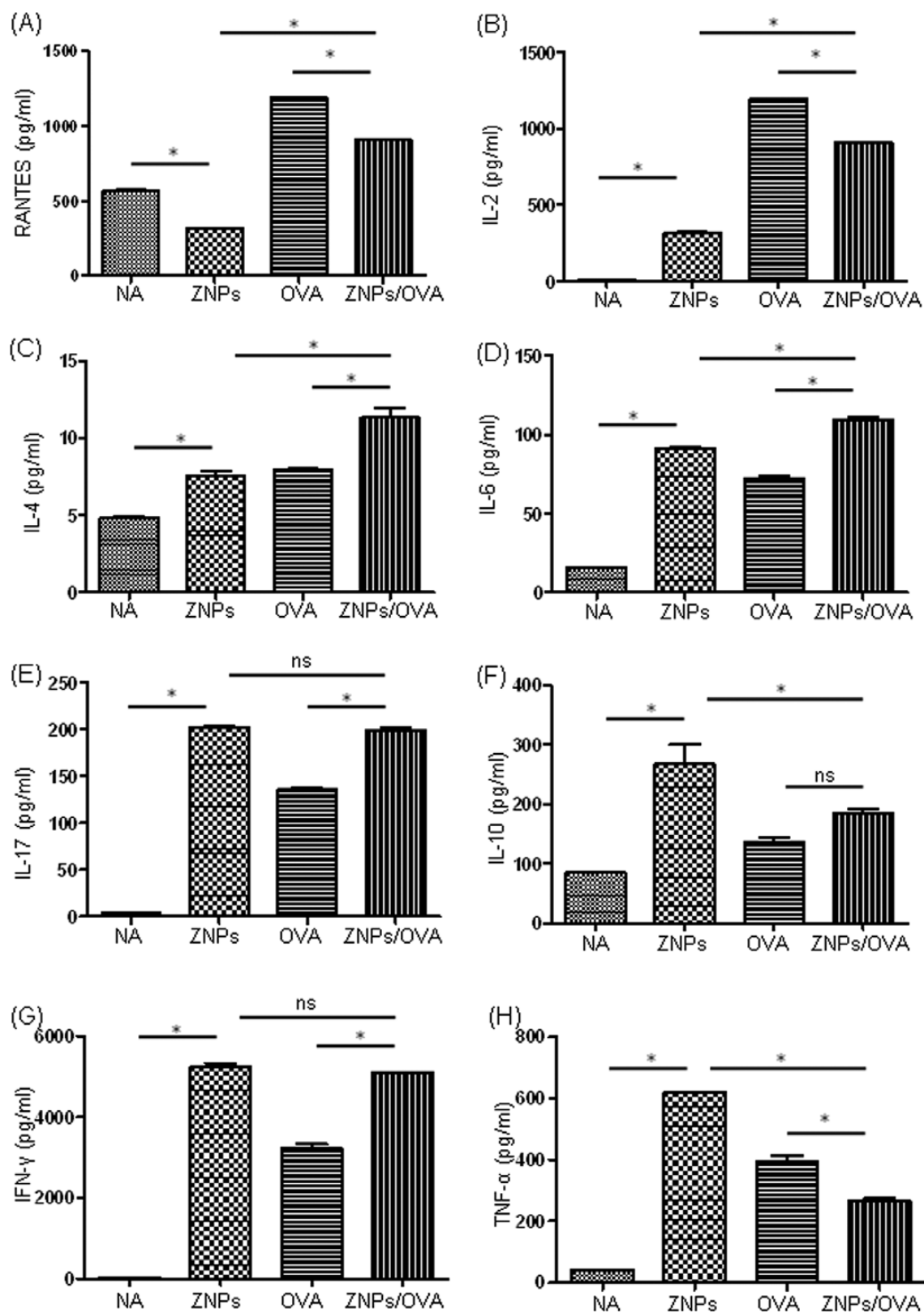


Fig. 9. The levels of cytokines in the splenocyte culture supernatants. (A) RANTES, (B) IL-2, (C) IL-4, (D) IL-6, (E) IL-17, (F) regulatory cytokine IL-10, (G) IFN- γ and (H) TNF- α in control (NA), ZNPs, OVA and OVA/ZNPs groups. Amounts were calculated from cytokine standards and absolute levels are given as pg ml⁻¹. Values represent means $\pm$ SEM of three replicates. Values represented are significantly different in NA versus ZNPs, ZNPs versus ZNPs/OVA and/or OVA versus ZNPs/OVA at * P < 0.05 and ns = non-significant.

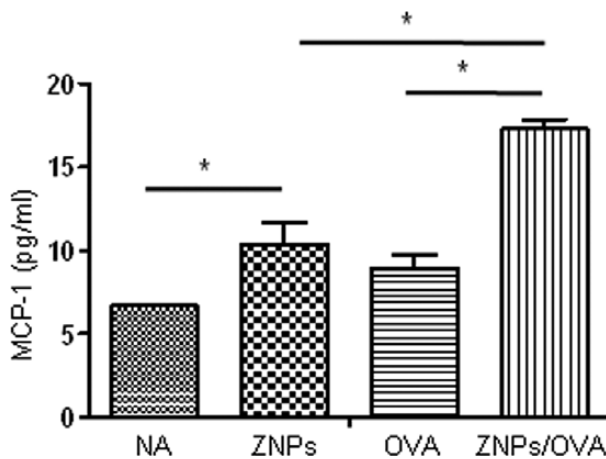


Fig. 10. MCP-1 level in the serum samples of control (NA), ZNPs, OVA and OVA/ZNPs groups. Values represent means $\pm$ SEM of three replicates. Values represented are significantly different in NA versus ZNPs, ZNPs versus ZNPs/OVA and OVA versus ZNPs/OVA at $*P < 0.05$.

monocytes to the site of damage/inflammation by secretion of MCP-1 (37–39). In order to demonstrate this, invasion of neutrophils in different organs was quantified by measuring MCP-1 release and MPO activity that demonstrated increased neutrophil infiltration into lungs and intestines of the ZNPs/OVA-treated group.

The extent of inflammatory effects can also be assessed by the degree of macrophage activation. Potent inflammatory responses induce extensive activation of macrophages. The processed antigens are transmitted to T and B cells for production of antibodies. Therefore, we postulated that APCs may also be involved in antibody production and found a significant increase in CD11b, MHC-I and MHC-II (>3-fold increase) molecules and the increasing levels of CD11b and MHC-II were more pronounced. It is known that IL-4 and IL-13 up-regulate the expression of the mannose receptor and MHC-II molecules by macrophages, which in turn stimulate endocytosis and antigen presentation, culminating in induction of selective chemokines, including macrophage-derived chemokines, which are believed to be involved in cell recruitment (40). Since macrophage activation for phagocytosis and antigen processing is the first step of antibody development, inflammation-primed macrophages play a major role in immune development. These responses may relate to their ability to cause inflammation, thereby intensifying the general reactions such as chemotaxis and activation of APCs to antigen exposure.

It has been observed that after OVA challenge in a mouse model mimicking allergic reactions, the increased IL-17⁺ cells in the lungs were mainly CD11b⁺ macrophages, instead of T cells or others (41). It is also known that macrophages rather than T_H17 cells are the main producer of IL-17 in allergic inflammation related to asthma. Therefore, we have assessed the expression of CD11b⁺ macrophages in peritoneal lavage and this corroborated our finding of an increase in CD11b⁺ expression in the ZNPs/OVA-treated group, as well as the OVA-treated group alone. The above process was negatively regulated by the T_H2 cytokine IL-10 and positively regulated by mast-cell-released mediators (41). This is in line with our result of IL-10 down-regulation.

TLR-4 signaling plays an important role in the mechanism of CD11b expression and up-regulation of CD11b is responsible for activation of polymorphonuclear cell adhesion and trans endothelial migration response; thus, TLR-4-induced up-regulation of CD11b may serve an important role in the innate immune response. During acute intestinal inflammation in inflammatory bowel diseases, TLR-4 expression is enhanced (42). Moreover, TLR-4 up-regulation could also result from the exposure of ligands other than LPS such as nanoparticles (43). We have found enhanced TLR-4 in the intestine of the ZNPs/OVA group. Previously, the activation of TLR-4 has been suggested to be required for optimal development of T_H2 responses (44). The interaction of ultrafine particles with TLRs provides a possible link between innate immune cell activation and allergic sensitization.

The inhibition of T_H1 responses by GATA-3 presumably is through inhibiting the expression of T-bet (45). Negative regulators of cytokine signaling, the SOCS proteins, play an important role in T_H2-mediated allergic responses through the control of the balance between T_H1 and T_H2 cells. SOCS-3 is predominantly expressed in T_H2 cells and they reciprocally inhibit the T_H1 differentiation processes.

Conclusions

The enhanced levels of specific IgE, IgG1, mast cell protease-1, T_H2 cytokines, eosinophils and mast cells in the intestine, the increased MPO activity, prominent anaphylactic symptoms and eruptive histopathological changes in the ZNPs/OVA group compared with the only OVA-sensitized group indicate the adjuvant property of ZNPs.

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