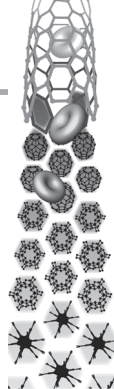


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Adjuvanticity and toxicity of cobalt oxide nanoparticles as an alternative vaccine adjuvant

Aim: There are very few adjuvants licensed for use in human vaccination, and alum-based adjuvants are the most widely used. Alum adjuvants predominantly boost Th2 immune responses and there is a need for new adjuvants that also stimulate Th1 immunity. We recently reported that cobalt oxide nanoparticles (Co_3O_4 NPs) stimulate Th1-type immune responses *in vivo*. Here, we exploited this property to examine whether Co_3O_4 NP could act as an adjuvant using the model antigen ovalbumin. **Materials & methods:** Female C57BL/6 mice were immunized subcutaneously twice with ovalbumin plus adjuvant (Co_3O_4 NPs or Imject® Alum) followed by intraperitoneal stimulation with soluble ovalbumin. **Results:** Co_3O_4 NPs induced a more balanced Th1- and Th2-type response, triggering higher specific Th1-dependent IgG2c production in addition to Th2-dependent IgG1 and less 'allergic' IgE production, and induced less inflammation at both the subcutaneous and intraperitoneal injection sites. **Discussion:** Co_3O_4 NPs could be a very useful adjuvant where both Th1 and Th2 responses are needed to clear pathogens.

Original submitted 22 August 2011; Revised submitted 28 February 2012; Published online 20 July 2012

KEYWORDS: adjuvant ■ alum ■ cobalt oxide nanoparticle ■ ovalbumin ■ toxicity

Vaccines are considered to be the ultimate immunological tool in the prevention of infectious disease by providing a long-lasting protective immunity with a good antibody response [1,2]. The role of the adjuvant is to enhance the specific response of the immune system to a minimal amount of the antigen supplied, through activation of antigen-presenting cells [3]. The ideal adjuvant would not be in itself antigenic and would boost immune responses, whilst not inducing overreaction of the immune system, which could harm the patient (e.g., inducing excess local inflammation, allergy or delayed-type hypersensitivity). Licensed adjuvants are very limited in number owing to the safety concerns surrounding them. Despite this, pain and inflammation at sites of injection are common side effects of the use of adjuvants in clinical vaccines.

The adjuvants most widely used in human vaccines are aluminum-containing adjuvants [4], such as alum, a mineral salt, usually made up as $\text{Al}(\text{OH})_3$ or $\text{Al}(\text{PO})_4$. Alum adjuvants are thus metal oxide particulates. Aluminum containing adjuvants induce humoral immunity predominantly by promoting Th2-type responses in mice, whilst having a poorer ability to stimulate Th1-dependent antibodies. Alum-based adjuvants do not usually induce strong cell-mediated immune responses particularly cytotoxic T-cell responses [4], although they have been reported to prime cytotoxic CD8 responses

in the presence of additional lipid adjuvant [5]. Therefore, alum-containing adjuvants are suitable for responses against protein antigens or inactivated organisms but not for inducing responses to intracellular pathogens [1–4]. Thus, alternative adjuvants are needed for diseases in which cell-mediated immune responses are important for prevention or cure.

Nanoparticles (NPs) have been expanded to a wide range of biomedical applications owing to their unique physicochemical properties compared with bulk chemicals [6]. Compared with bulk chemicals, NPs have greater surface area and high protein-binding affinity by electrostatic interaction [7]. NPs can also penetrate deep into tissue and enhance cellular uptake, as well as escape lysosomal compartments [7–9]. These unique properties have led to NP being considered as potential adjuvants [10,11]. The size of NPs appear to affect adjuvant ability as smaller NPs have been shown to produce greater antibody and cellular immune responses [12]. However, most of the NP candidates for vaccine adjuvants are organic substances [13] and few studies have tested inorganic NPs. In murine asthma models, multiwalled carbon nanotubes [14], diesel exhaust particles [15] and nano-sized ultrafine carbon black [16] were reported to increase serum IgE levels by an adjuvant-like mechanism. In comparison with data reported using alum-containing adjuvants [17,18], nano-sized carbon

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black showed a similar adjuvant effect with IgG1 and IgE increases [16].

In our previous work, we found both nickel oxide NPs (NiONPs) and cobalt oxide NPs (Co_3O_4 NPs) were capable of inducing a Th1 response in the lungs of female Wistar rats [19]. As so very few adjuvants are capable of eliciting a Th1 response it was considered that one of these NPs could be utilized to improve Th1 immunity against a model antigen, ovalbumin (OVA). In this study, we selected Co_3O_4 NPs as a candidate adjuvant because Co_3O_4 NPs produced a Th1 response without the severe delayed-type hypersensitivity pathology seen with NiONPs [19]. The aim of this study was to determine whether Co_3O_4 NPs could be suitable for use as an adjuvant by inducing a balanced Th1 and Th2 response to the model antigen OVA, given subcutaneously to female C57BL/6 mice. To this end we compared anti-OVA responses induced by Co_3O_4 NPs to those induced by a commercially available aluminum-containing adjuvant. The adjuvant chosen was Imject® Alum (I-Alum; Thermo Scientific, Cramlington, UK), a metal particulate adjuvant widely used for murine experiments, which contains a 50/50 mixture of aluminum hydroxide and magnesium hydroxide and inactive stabilisers. In addition to OVA-specific responses, toxicity was also assessed at the injection site and cytotoxicity for antigen-presenting cells was evaluated using a mouse macrophage cell line.

Materials & methods

All chemicals and reagents were purchased from Sigma-Aldrich (Poole, UK), unless otherwise stated.

■ Characterization & dispersion of particles

The size of Co_3O_4 NPs (Nanostructural and Amorphous Materials, TX, USA) and I-Alum was measured using transmission electron microscopy (JEM-1200EX II, JEOL, Tokyo, Japan). The hydrodynamic sizes of particles were measured using a particle size analyzer (90Plus/BI-MAS; Brookhaven Instruments Corporation, NY, USA) in the presence of various concentrations of a dispersion medium. For *in vivo* study, hen egg OVA (grade V) was used as a dispersion medium for Co_3O_4 NPs + OVA (NP-OVA) and I-Alum + OVA (I-Alum-OVA), whilst heat-inactivated serum (final concentration: 5 %) collected from healthy C57BL/6 mice was used for NPs alone. The

levels of endotoxin in the particle suspensions were measured using a Limulus Amebocyte Lysate assay (Cambrex, MD, USA). The solubility of Co_3O_4 NPs and I-Alum was tested in the acidic artificial lysosomal fluid (pH 5.5) or basic artificial interstitial fluid (pH 7.4) according to the previously described method [19].

■ Rationale for dose selection

Based on our previous study, we selected the dose of I-Alum as 2.0 mg per mouse [17]. In contrast, the dose of Co_3O_4 NPs was selected as 25 µg per mouse based on the inflammogenicity in the lung; instillation of 419 µg/rat of Co_3O_4 NPs showed acute neutrophilic inflammation and chronic lymphocytic/neutrophilic inflammation with alveolar lipoproteinosis [19].

■ Cytotoxicity assay

Mouse macrophage cell line, RAW264.7, was cultured at 37°C with 5% CO_2 in DMEM with L-glutamine, antibiotics (penicillin and streptomycin) and 10% heat-inactivated fetal bovine serum. Cells were detached using accutase and seeded at 5×10^5 in 12-well plates (Corning, NY, USA) for experiments. The cytotoxic effects of NPs and alum were measured using a lactate dehydrogenase assay kit (Roche Applied Science, Sussex, UK) according to the manual instruction. As benchmark particles, TiO_2 NPs (30.5 ± 1.8 nm; Nanostructural and Amorphous Materials) were used. NP or I-Alum solutions with or without OVA (1%) were treated to RAW264.7 cells for 24 h. Each NP solution was tested at concentrations of 10, 30, 100 and 500 µg/ml, whilst I-Alum was tested at concentrations of 80, 240 and 800 µg/ml. After incubation, cell-free culture supernatants were centrifuged at $17000 \times g$ for 20 min to get rid of particles. The cytotoxicity was calculated as a percentage compared with the positive control (0.1% Triton-X).

■ Animal immunization

Six-week old female C57BL/6 mice were obtained from Harlan Laboratories (Hillcrest, UK). All animals were maintained and handled under a specific license granted by the UK Home Office to one of the authors that ensures humane treatment and alleviation of suffering in all animal experiments. After 1 week to acclimatize, mice (five mice per group) were immunized twice at 2-week intervals. Each mouse was sensitized by subcutaneous injection at the base of tail with a 100-µl dose containing 25 µl phosphate-buffered solution (PBS), 50 µl

(100 µg) endotoxin-free OVA (Worthington Biochemical Corporation, NJ, USA), and 25 µl of adjuvant (I-Alum: 2.0 mg; Co₃O₄ NPs: 25 µg). PBS (100 µl) and Co₃O₄ NPs (25 µg in PBS with 5% mouse serum) were used for negative controls. Then, 50 µg of OVA in 100 µl of PBS was injected into the peritoneum as an antigen stimulation. At day 7 after the second sensitization, blood was collected from the tail vein. After 7 days since intraperitoneal antigen administration, the blood was collected by cardiac puncture and peritoneal lavage was performed three-times using 2 ml of 0.9% saline. The first lavage fluid was kept for immunoglobulin and cytokine ELISA, whilst cells from three lavages were pooled for counting cells. The site of injection was also fixed with 10% neutral-buffered formalin for histological analysis.

■ Immunoglobulin ELISA

Blood samples collected 1 week after immunization and 1 week after intraperitoneal antigen stimulation were analyzed for the presence of OVA-specific immunoglobulins of IgA, IgE, IgG1, IgG2c (formerly known as IgG2a^b) and IgM subclasses. Serum and peritoneal lavage samples were diluted as appropriate in PBS with 1% bovine serum albumin. Biotin-conjugated secondary antibodies were diluted as follows: anti-IgA – 1:1000; anti-IgG1 – 1:8000; anti-IgG2c – 1:1000; anti-IgM – 1:1000; anti-IgE – 1:250. Before measuring IgE, serum was incubated for 1 h with protein G-coupled beads to remove IgG [17,18].

■ Cytokine ELISA

To evaluate the cytokine profile, Th1-type cytokine (IFN-γ), Th2-type cytokines (IL-10 and IL-13) and macrophage-derived proinflammatory cytokine (IL-1β) were tested in the peritoneal lavage. All kits were purchased from R&D Systems (Abingdon, UK) and cytokines were measured according to the manufacturer's instruction.

■ Differential cell counts of peritoneal lavage

The total cell number in lavage fluid was assessed by use of a cell nucleus counter (Chemometec, Surrey, UK). Cells were cytopspined onto glass slides (Thermo Scientific), fixed with methanol and stained with Diff-Quik (Raymond Lamb, Eastbourne, UK). Approximately 300–500 cells per slide were counted under a light microscope according to their morphology.

■ Lymphocyte proliferation assay

Spleens were removed from mice and single-cell suspensions made by passing through 40-µm filters. Cells were layered onto lympholyte-M™ (Fisher Scientific, Loughborough, UK) density gradient separation medium and spun at 2000 × g for 15 min to remove dead cells, erythrocytes and granulocytes. The mononuclear cell layer at the interface was removed, washed and resuspended in tissue culture medium (Roswell Park Memorial Institute medium 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol; Sigma-Aldrich). Viable cells were counted and triplicate cultures containing 4 × 10⁵ cells in 200 µl containing no or 10 µM endotoxin-free OVA were plated in 96-well plates. Cultures were incubated for 48 h and 0.5 µCi ³H-thymidine was added to each well overnight. Cultures were harvested and incorporated thymidine counted in a betaplate scintillation counter (Wallac UK, Milton Keynes, UK). Supernatants from parallel nonlabelled cultures were harvested at 72 h for cytokine analysis.

■ Histology

Paraffin-embedded formalin-fixed tissues from the injection site were cut into 3 µm sections and stained with hematoxylin and eosin.

■ Statistical analysis

Data were analyzed with GraphPad Prism Software (Version 5, GraphPad Software Inc., CA, USA). In order to compare treatment groups, one-way analysis of variance with *post hoc* Tukey's pairwise comparisons was applied. A *p* < 0.05 was considered to be statistically significant.

Results

■ Physicochemical properties of particles

Co₃O₄ NPs showed spherical shapes and the primary size was 18.4 ± 5.0 nm. I-Alum showed more heterogeneous shapes and the average size was 88.7 ± 32.4 nm for length and 28.9 ± 19.7 nm for width (FIGURE 1). Endotoxin levels of both particles were below the detection limit (0.1 EU/ml). Co₃O₄ NPs showed large agglomerates without a dispersion medium but small agglomerates with OVA as a dispersant (TABLE 1). I-Alum showed small agglomerates even without dispersion medium. Most I-Alum was dissolved within 3 days in the acidic artificial lysosomal fluid (pH 5.5), whilst minimally dissolved in the artificial interstitial fluid (pH 7.4) [CHO W-S ET AL., UNPUBLISHED DATA].

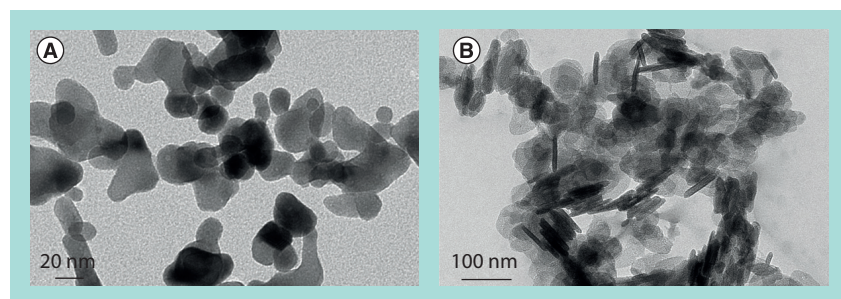


Figure 1. Electron microscopic appearance of adjuvants used. The shape and size of (A) Co_3O_4 nanoparticles and (B) Imject® Alum. Particles were dispersed in distilled water and measured by transmission electron microscopy.

Co_3O_4 NP showed minimal dissolution in both conditions [CHO W-S ET AL., UNPUBLISHED DATA].

■ *In vitro* cytotoxicity

I-Alum was by far the most toxic of the NPs tested on mouse macrophages (FIGURE 2). When particles were dispersed with OVA, particles were more toxic.

■ *In vivo* immunization

OVA-specific immunoglobulin levels in the serum after sensitization

IgG1 was the only immunoglobulin subclass measurable at this stage. FIGURE 3 shows that both I-Alum-OVA and NP-OVA induced IgG1 production, although I-Alum-OVA was more potent than NP-OVA. Levels of OVA-specific immunoglobulins induced by NP alone were similar to the vehicle control (PBS).

OVA-specific immunoglobulin levels in the serum after intraperitoneal antigen stimulation

Immunoglobulin levels after intraperitoneal antigen stimulation demonstrated a wider array of responses. Both NP-OVA and I-Alum-OVA produced increases in the IgG1 response although again I-Alum-OVA showed higher responses than NP-OVA (FIGURE 4A). The IgG1 levels in both NP-OVA and I-Alum-OVA groups showed a marked increase when compared with those of postsensitization. In addition, NP-OVA increased in IgG2c levels (FIGURE 4B). I-Alum-OVA induced higher IgE, IgM and IgA levels than NP-OVA (FIGURE 4C–4E).

Splenocyte proliferation after intraperitoneal antigen stimulation

Spleen mononuclear cells were stimulated *in vitro* with OVA 7 days after intraperitoneal antigen administration. FIGURE 4F shows that splenocytes from both I-Alum-OVA and NP-OVA immunized mice proliferated.

OVA-specific immunoglobulin levels in the peritoneal lavage after intraperitoneal antigen stimulation

Both NP-OVA and I-Alum-OVA showed increases in the IgG1 levels (FIGURE 5). I-Alum-OVA showed higher IgG1 levels compared with NP-OVA. Other immunoglobulins (IgG2c, IgE, IgA and IgM) were detected only at very low concentrations, which were similar to the PBS group [CHO W-S ET AL., UNPUBLISHED DATA].

Cytokine expression in the peritoneal lavage fluid

The peritoneal lavage fluid collected from the mice was also used to measure cytokine expression. Levels of IFN- γ were significantly increased by the NP-OVA whilst other groups showed no significant responses (FIGURE 6A). Levels of IL-1 β were significantly increased by I-Alum-OVA only (FIGURE 6B). IL-10 and IL-13 showed no significant changes compared with controls [CHO W-S ET AL., UNPUBLISHED DATA].

Differential cell counts in peritoneal lavage

Number of total cells and lymphocytes was significantly increased by both NP-OVA and I-Alum-OVA whilst NP alone was comparable to vehicle control (FIGURE 7A & 7B). The number of total granulocytes was significantly increased by I-Alum-OVA only (FIGURE 7C).

Inflammation at the injection site

Histology samples taken from the injection site at 7 days after intraperitoneal antigen stimulation provided sharply contrasting results between the two particles tested. The I-Alum-OVA treatment group showed marked inflammation and necrosis with no obvious particle deposition, whilst the NP-OVA and

Table 1. Hydrodynamic size and polydispersity of particles 24 h after dispersion (mean $\pm$ standard deviation).

Dispersion	Co_3O_4 NP		Imject® Alum	
	Hydrodynamic size	Polydispersity	Hydrodynamic size	Polydispersity
Saline	943.0 $\pm$ 251.1	0.15 $\pm$ 0.01	218.9 $\pm$ 119.2	0.23 $\pm$ 0.06
Ovalbumin 0.1%	90.3 $\pm$ 17.9	0.16 $\pm$ 0.02	241.2 $\pm$ 62.1	0.29 $\pm$ 0.03
Ovalbumin 1%	99.8 $\pm$ 32.6	0.20 $\pm$ 0.02	126.2 $\pm$ 36.9	0.19 $\pm$ 0.05

NP alone group showed minimal inflammation with surrounding particle deposition (FIGURE 8).

Discussion

The safety of adjuvants used on humans is a key issue in their development. Recent studies have shown that the licensed adjuvant activates human monocytes and macrophages [20], suggesting that what would be deemed as toxicity is potentially a part of the mechanism by which alum adjuvant works. In this study, we hypothesized that Co_3O_4 NPs may produce a balanced Th1 and Th2 response with less toxicity than alum, suggesting that they may be a safer option.

Co_3O_4 NPs showed relatively homogenous spherical shape of particles. Interestingly, I-Alum was more heterogeneous and less than 100 nm in both dimensions. The heterogeneous shape might be due to the composition of I-Alum comprising a mixture of aluminum hydroxide and magnesium hydroxide. Therefore, both Co_3O_4 NPs and I-Alum were within the definition of NPs and classified as metal oxide NPs [21,22]. Since previous studies have shown that size matters in adjuvanticity, optimal dispersion of NPs might be beneficial [12]. OVA acted as an excellent dispersant for both particles. Co_3O_4 NPs showed small agglomerates when dispersed with OVA, which form a protein corona providing repulsive force between particles [23]. In contrast to Co_3O_4 NPs, I-Alum was relatively well-dispersed in saline, which might be because of the stabilizers added during manufacturing.

Adjuvants are believed to work by activating antigen-presenting cells but there is little information on their toxicity for such cells. Using a murine macrophage cell line, the lactate dehydrogenase cytotoxicity study showed that I-Alum by itself was approximately six-times more toxic than an equivalent amount of Co_3O_4 NPs. Dispersal in OVA showed increased toxicity of both Co_3O_4 NPs and alum, which might be due to the cytotoxicity of OVA itself or to facilitating recognition and phagocytosis of particles by forming a protein corona.

The postsensitization data showed that both I-Alum–OVA and NP–OVA increased serum OVA-specific IgG1 levels (which is a marker for Th2 response [3]), whilst other immunoglobulins were not increased. However, the potential for IgG1 production by I-Alum–OVA was approximately three-times higher than that of NP–OVA. The postintraperitoneal antigen stimulation data showed that a T-cell response

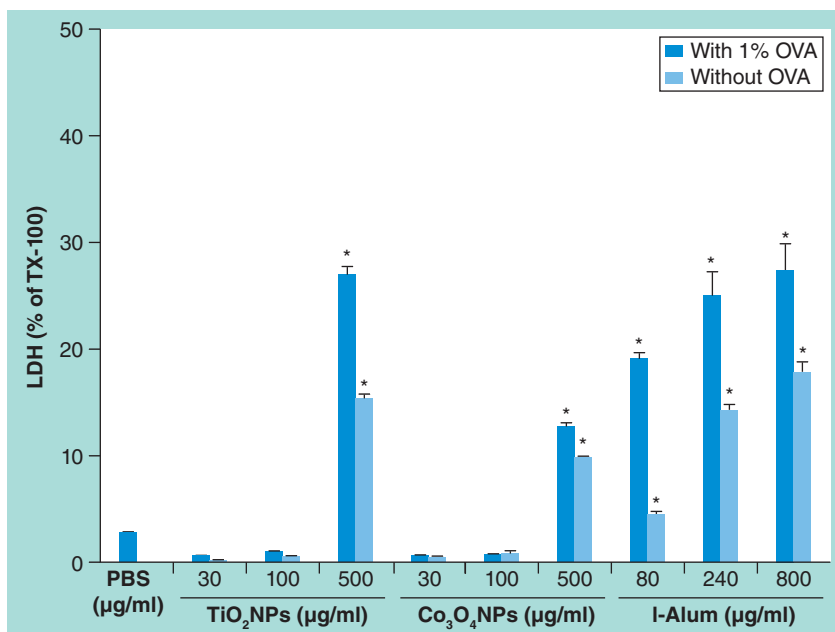


Figure 2. Cytotoxicity of nanoparticles or Imject® Alum to RAW264.7 cells. Particles were added to RAW264.7 cells for 24 h with or without 1% OVA. Levels of LDH were measured for cytotoxicity. Note that I-Alum showed around six-times more cytotoxicity compared with Co_3O_4 NP and OVA increased the toxicity of particles. Data are mean $\pm$ standard error of the mean ($n =$ four per group). The cytotoxicity data of particles treated with 1% OVA or without OVA were compared with their respective controls.

* $p < 0.001$.

I-Alum: Imject® Alum; LDH: Lactate dehydrogenase; NP: Nanoparticle; OVA: Ovalbumin; PBS: Phosphate-buffered saline.

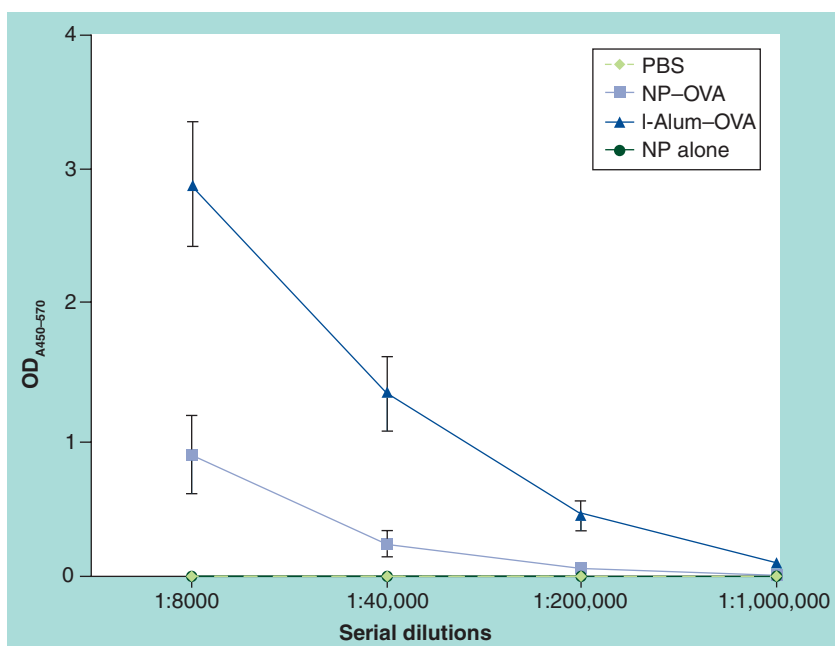


Figure 3. Ovalbumin-specific IgG1 levels in serum 7 days after second subcutaneous sensitization. Other immunoglobulins (IgG2a, IgE, IgA and IgM) showed no increases [CHO W-S ET AL., UNPUBLISHED DATA]. Data are mean $\pm$ standard error of the mean ($n =$ five per group).

I-Alum: Imject® Alum; NP: Nanoparticle; OD: Optical density; OVA: Ovalbumin; PBS: Phosphate-buffered saline.

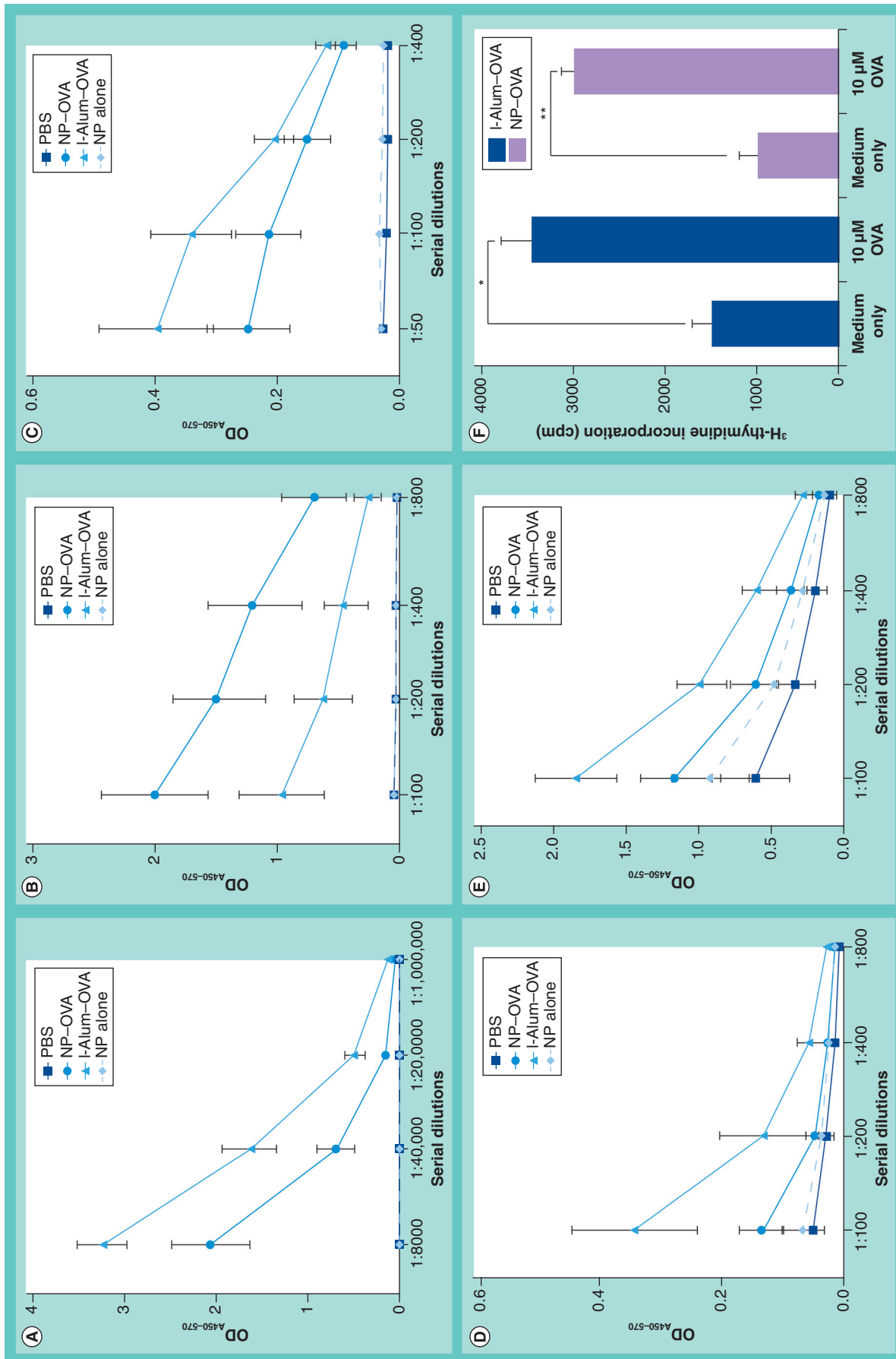


Figure 4. Ovalbumin-specific immunoglobulin levels present in serum 7 days after intraperitoneal stimulation with ovalbumin. Immunoglobulins tested for were (A) IgG1, (B) IgG2a, (C) IgE, (D) IgA and (E) proliferation of spleen cells from mice immunized with I-Alum-OVA or NP-OVA 7 days after intraperitoneal stimulation with OVA. ³H-thymidine incorporation of cells cultured with medium only or in the presence of 10 μM OVA. *p < 0.05; **p < 0.01. I-Alum: Inject® Alum; NP: Nanoparticle; OD: Optical density; OVA: Ovalbumin; PBS: Phosphate-buffered saline.

was generated, confirmed by proliferation of spleen cells cultured with OVA *in vitro*. Further boosted immunoglobulin responses were also seen in mice immunized with either adjuvant. The presence of antigen-specific class-switched immunoglobulins (IgG and IgE) in serum indicate that both Th1 and Th2 OVA-specific CD4⁺ T lymphocytes have been activated by OVA after sensitization with the antigen using Co₃O₄ NPs as an adjuvant. In serum I-Alum-OVA produced mainly Th2-type (IgG1) and allergic antibody responses (IgE), whilst NP-OVA produced a more balanced Th1- (IgG2c) and Th2- (IgG1) type response with much less IgE. As many infections require Th1 responses and as well as antibodies for proper clearance and induction of immunological memory, Co₃O₄ NPs as an adjuvant may be more likely to promote the induction of more efficient immune responses, and less likely to induce unwanted allergic side effects. This was further supported by the postintraperitoneal antigen stimulation peritoneal lavage fluid analysis. Th2-dependent IgG1 levels were significantly increased by NP-OVA and I-Alum-OVA with the same pattern as seen in serum. Peritoneal lavage IFN- γ , a Th1-type cytokine [24], was increased only in the NP-OVA immunized group. The differential cell counts of the peritoneal lavage fluid showed that both NP-OVA and I-Alum-OVA induced lymphocytic inflammation, which is important

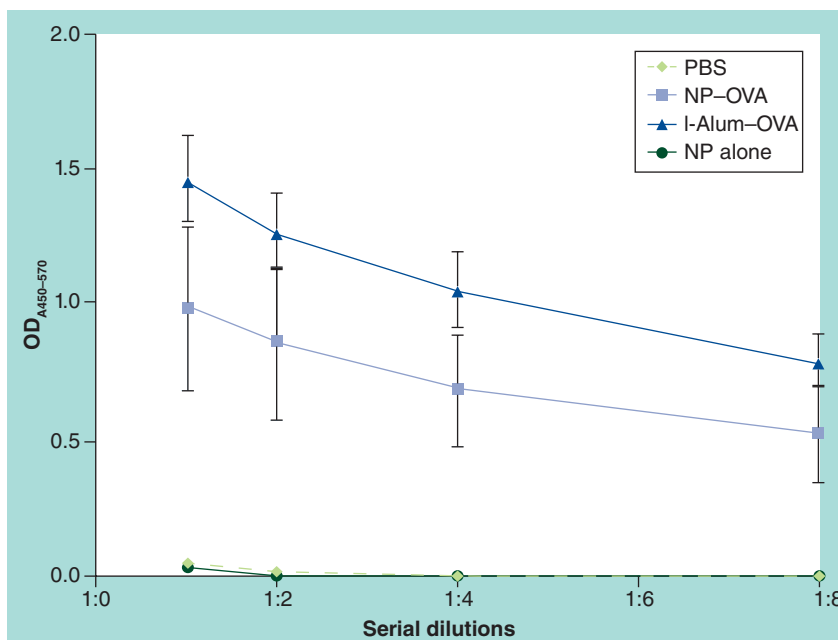


Figure 5. Ovalbumin-specific IgG1 levels in peritoneal lavage fluid 7 days after intraperitoneal stimulation with ovalbumin. Other immunoglobulins (IgG2a, IgE, IgA and IgM) showed no increases [CHO W-SET AL., UNPUBLISHED DATA]. Data are mean $\pm$ standard error of the mean (n = five per group). I-Alum: Imject® Alum; NP: Nanoparticle; OD: Optical density; OVA: Ovalbumin; PBS: Phosphate-buffered saline.

for adjuvanticity [5], but NP-OVA induced far less granulocytic inflammation. This granulocytic inflammation by I-Alum-OVA was consistent with the increased IL-1 β in the peritoneal lavage, which is one of the macrophage-derived

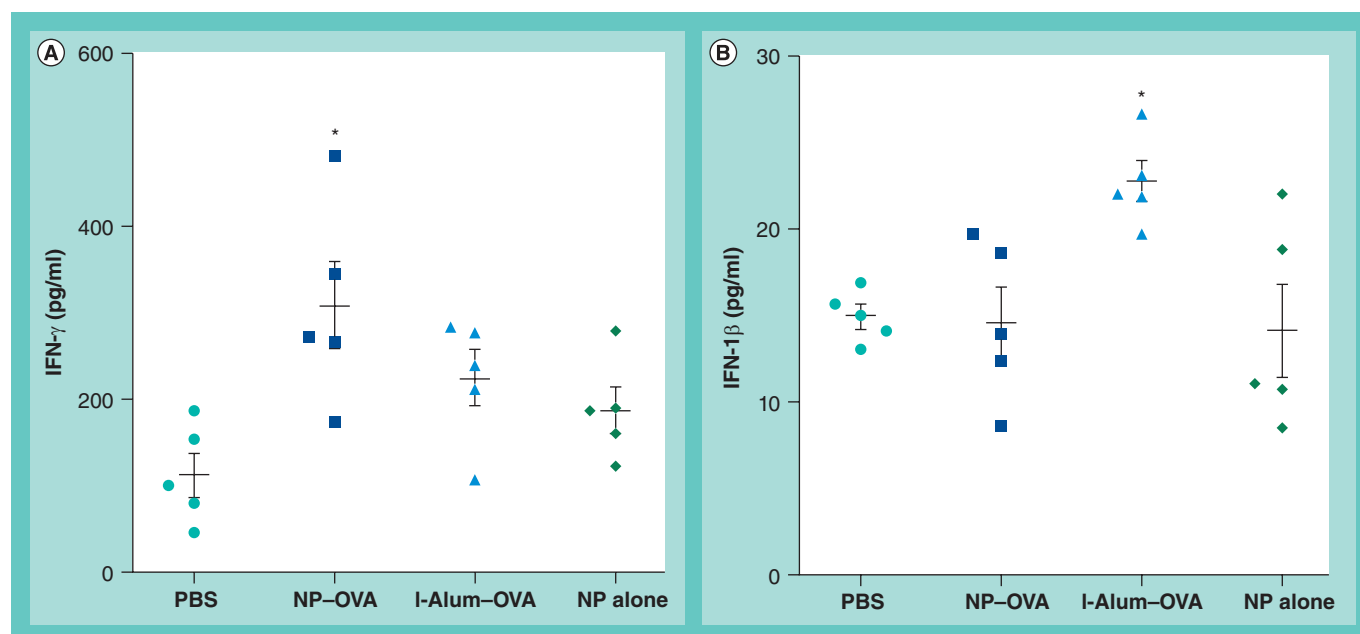


Figure 6. Levels of cytokine in the peritoneal lavage fluid collected 7 days after intraperitoneal antigen administration. (A) IFN- γ ; (B) IL-1 β . Data are mean $\pm$ standard error of the mean (n = five per group). *p < 0.05 compared with the PBS treatment group. I-Alum: Imject® Alum; NP: Nanoparticle; OVA: Ovalbumin; PBS: Phosphate-buffered saline.

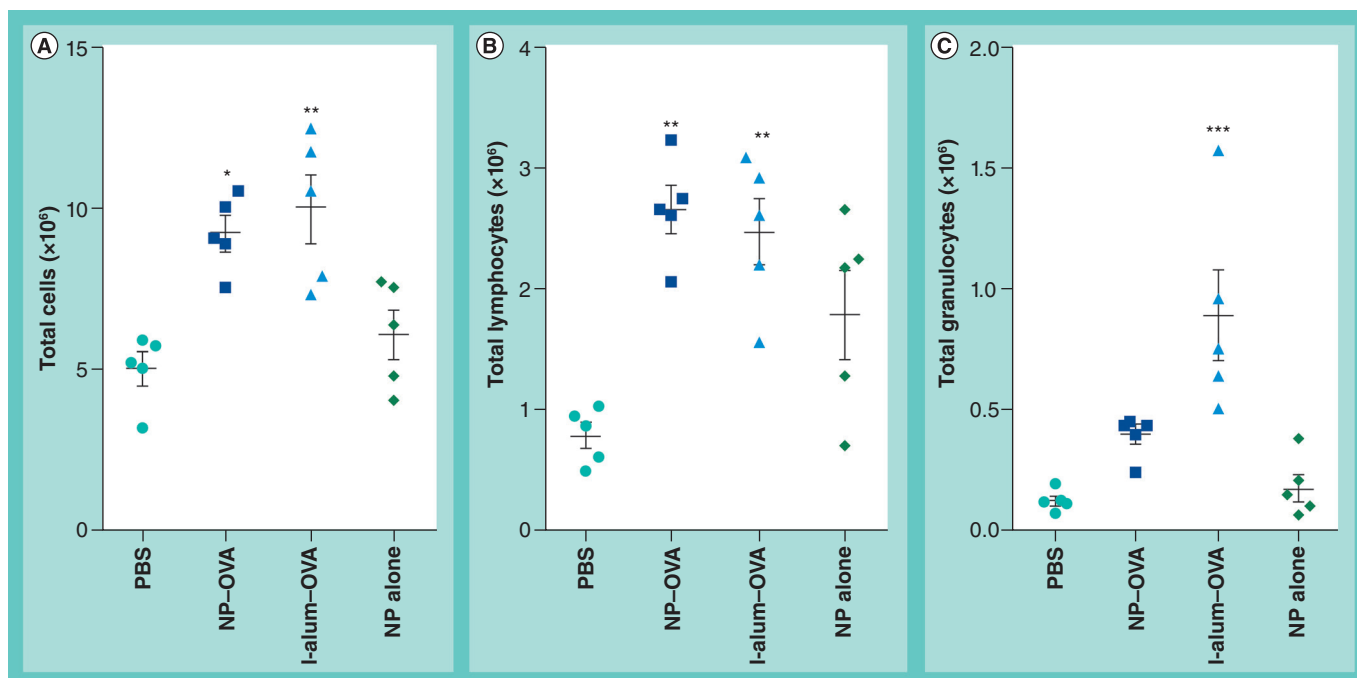


Figure 7. Differential cell counts of the peritoneal lavage fluid collected 7 days after intraperitoneal antigen administration. Data are mean $\pm$ standard error of the mean ($n =$ five per group). (A) Total cells; (B) total lymphocytes; and (C) total granulocytes present in peritoneal lavage fluid.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with the PBS treatment group.

I-Alum: Imject® Alum; NP: Nanoparticle; OVA: Ovalbumin; PBS: Phosphate-buffered saline.

proinflammatory cytokines. The pattern of response induced by I-Alum–OVA is consistent with that seen in other studies [25,26].

At 7 days after intraperitoneal antigen administration at the doses used, I-Alum induced prolonged massive inflammation and necrosis in the subcutaneous and muscular layer of the injected skin, whilst Co_3O_4 NPs showed mild inflammation. The greater inflammation induced by I-Alum compared with Co_3O_4 NPs was consistent with increased peritoneal IL-1 β secretion and the number of granulocytes recruited to the peritoneal cavity by I-Alum indicating inflammatory responses at both the subcutaneous and intraperitoneal antigen administration sites. In addition, the I-Alum induced more allergic anti-OVA IgE serum antibodies. This provides a more rounded result with Co_3O_4 NPs than a previous report, where TiO_2 NPs showed a higher IgE response than $\text{Al}(\text{OH})_3$, although in that case $\text{Al}(\text{OH})_3$ produced the higher granulocyte recruitment concurring with our data [27]. In addition, Co_3O_4 NPs showed a more balanced Th1 and Th2 response even with 80-times less mass administered than I-Alum. The absence of cell death and inflammation at the injection site of the NP–OVA group indicates that it will cause less local pain. In addition, if the NP adjuvant

could be used to vaccinate food animals, less cell death and inflammation at the injection site would mean less hide and meat spoilage and may have improved economic benefits.

Within 3 days of incubation, most of the I-Alum was dissolved in the acidic (pH 5.5) solution, which is the same pH as the lysosomal fluid of alveolar macrophages [28], whilst there was minimal dissolution in the basic (pH 7.4) solution. However, Co_3O_4 NPs showed minimal dissolution in both conditions. Compositional ions released in the acidic lysosomal condition would be likely to play a role in cell death and inflammation for high-solubility NPs such as CuONPs [29] and ZnONPs [30]. Therefore, Al^{3+} dissolved in the lysosomes of phagocytic cells might contribute to cell death and further inflammation by a Trojan Horse-type mechanism [31]. Co_3O_4 NPs had not changed in solubility and this was consistent with persistence at the injection site. This suggests that the Co_3O_4 NPs are very insoluble and stable. There is a concern regarding the solubility of Co_3O_4 NPs, as cobalt ions are being released very slowly into cells and their long-term effect is unknown [19]. Cobalt poisoning is not common but it can occur. There are reports of hip replacements (which contain cobalt and chromium) that corrode over time and release metal ions into the body causing

severe illness [32]. This seems an unlikely side effect here for Co₃O₄NPs due to their small dose and volume of injection and insoluble nature. In our previous study, deposition of Co₃O₄NPs inside of the lung causes delayed-type hypersensitivity and pulmonary alveolar lipoproteinosis [19]. However, considering the lung has a much more active immune response than the skin and muscle, the subcutaneous side effects might be more limited, and indeed, only mild inflammation was produced in the injection site at 5 weeks after two injections. However, if multiple repeated vaccinations were to be administered, a build-up of Co₃O₄NPs and release of cobalt ions may cause health problems. Accumulation of the Co₃O₄NPs within the subcutaneous and muscular layers indicates that Co₃O₄NP may provide a continuous source of desorbed antigen which is one of the modes of actions proposed for alum adjuvants [33].

The comparison between I-Alum and Co₃O₄NPs is suitable for measuring a response in mice. I-Alum is not licensed for human use and has a different physicochemistry to clinically approved products [33]. To be able to fully evaluate Co₃O₄NPs in a long-term study as a prospective human adjuvant, further studies are required; however, the authors believe that Co₃O₄NPs shows great promise in inducing both Th1- and Th2-mediated responses.

Conclusion

In conclusion, Co₃O₄NPs stimulated less allergic antibody production and *in vivo* inflammation (at both subcutaneous and intraperitoneal antigen administration sites) than a standard dose of the alum-containing adjuvant I-Alum. Together with the evidence that they also produced lower *in vitro* toxicity whilst stimulating both Th1 and Th2 *in vivo* antibody responses, this indicates

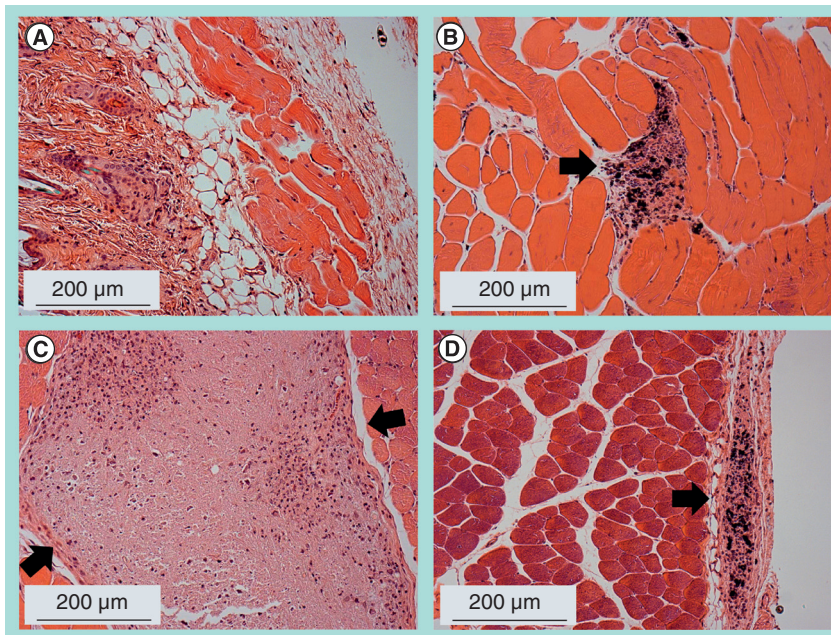


Figure 8. The representative histology of injection site 7 days after intraperitoneal antigen administration. (A) Phosphate-buffered saline; **(B)** nanoparticle (NP)–ovalbumin (OVA); **(C)** Imject® Alum–OVA; **(D)** NP alone. Note that NP–OVA and NP alone group showed mild inflammation (arrow) along with the deposition of the NPs (black spots), whilst Imject Alum–OVA showed massive inflammation and necrosis (arrow).

that Co₃O₄NPs would be suitable for use as a vaccine adjuvant.

Future perspective

From the viewpoint of the authors, there are strong reasons for developing novel adjuvants to replace alum-based adjuvants. Nanotechnology can meet this call because of the unique physicochemical properties of NPs. Modulation of the immune system using nanomaterials and development of novel adjuvants inducing optimal Th1 and Th2 response with minimal toxicity and inflammation might be possible in the near future.

Executive summary

Background

- Co₃O₄ nanoparticles (NPs) used as an adjuvant induced a balanced Th1 and Th2 response to model antigen with little evidence of allergy, inflammation or toxicity.

Ovalbumin-specific immunoglobulin levels in the serum after subcutaneous sensitization & intraperitoneal antigen administration

- Co₃O₄NPs induced specific serum Th1 (IgG2c) and Th2 (IgG1), but not IgE antibodies.

Cytokine expression in the peritoneal lavage fluid

- Co₃O₄NPs induced IFN-γ a marker for Th1 response in the peritoneal lavage.

Differential cell counts in peritoneal lavage & inflammation at the injection site

- Co₃O₄NPs did not induce granulocytic inflammation at the site of subcutaneous sensitization or in the peritoneal cavity following antigen stimulation.

Conclusion

- Co₃O₄NPs would be suitable for use as a vaccine adjuvant.

Financial & competing interests disclosure

Financial support was provided by the University of Edinburgh and the Medical Research Council of the United Kingdom (MRC G0701323). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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