NiO and Co$_3$O$_4$ Nanoparticles Induce Lung DTH-Like Responses and Alveolar Lipoproteinosis

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Abstract

Lung exposure to metal oxide nanoparticles (NP) comprising soluble metal haptens may produce Type 1 helper T cells (Th1) and Type 17 helper T cells (Th17) associated delayed-type hypersensitivity (DTH) responses and pulmonary alveolar proteinosis (PAP).

In order to study this, haptenic metal oxide NP (NiO, Co3O4, Cr2O3, and CuO) were instilled into the lungs of female Wistar rats and the immuno-inflammatory responses were assessed at 24 hours and 4 weeks post-instillation. Primary culture of alveolar macrophages from Wistar rats was used to evaluate the effect of the NP on the ability to clear surfactant.

NiONP induced chronic interstitial inflammation and pro-inflammatory Th1 and Th17 immune responses characterized by increases in the cytokines MCP-1/CCL2, IL-12+p40, IFN-γ, and IL-17A, whilst similar pathological responses induced by Co3O4NP were associated with increases in MCP-1/CCL2 and IL-12+p40. However, neither Cr2O3NP nor CuONP elicited immuno-inflammatory reactions. PAP was induced by both NiONP and Co3O4NP at the chronic phase. PAP was associated with over-production of surfactant by proliferation of type II cells and impaired clearance of surfactant by macrophages.

These findings have implications for the risk management of occupational NP exposure and provide evidence that haptenic metal oxide NP can induce chronic progressive lung immune responses via a DTH-like mechanism.

Keywords: delayed type hypersensitivity, hapten, metal oxide nanoparticles, pulmonary alveolar proteinosis, rat
**Introduction**

Metal oxide nanoparticles (NP) are known to release metal ions which can contribute to their toxicity [1, 2]. Some metal ions are known to induce contact dermatitis via delayed-type hypersensitivity (DTH) mediated by a cluster of differentiation 4 (CD4) T lymphocyte-dependent inflammatory immune response that involve Th1 and/or Th17 effector CD4 T cells [3-5]. Those metal ions triggering immune responses are regarded as haptens, small molecular weight molecules which are not themselves antigenic, but when bound to a host protein elicit sufficient structural change in the protein and its processed peptides that they can be recognized as non-self by T lymphocytes [6]. This may lead to the development of autoimmunity [7]. Metal haptens include Ni, Co, Cr, and Cu, and 10 – 15% of the general population shows allergic response to at least one of these metal ions [8]. The use of medical metal implants leading to raised levels of metal ions in serum has raised concern about metal hypersensitivity or carcinogenicity in patients [9].

Currently, large scale production of metal oxide NP for industrial, cosmetic, and medical imaging purposes raises the likelihood of inhalation exposure in occupational, consumer, and clinical settings. Although metal haptens can cause DTH in the skin, it is not known if, following deposition in the lungs, NP containing haptenic metals, can induce similar immunoinflammatory effects there. Other human lung pathology associated with inhalation of particles includes pulmonary alveolar proteinosis (PAP) characterized by accumulation of surfactant proteins and lipids in the alveoli [10, 11]. Our previous study with NiONP was the first to report induction of PAP by NP exposure [12]. PAP in humans can also be caused by an autoimmune response to GM-CSF [13]. However, whether other haptenic metal NP can cause PAP and by which underlying mechanisms is not known.
In our previous study, we tested a number of different NP for lung toxicity and found that they induced unique inflammatory footprints including neutrophilic, eosinophilic, and mononuclear inflammation [12]. In particular, we found that NP containing the metal hapten, nickel (NiONP), induced lymphocytic inflammation and PAP at the 4 wks post-instillation into rat lungs. Here, a panel of NP was assembled comprising the oxides of haptenic metals - NiONP, Co$_3$O$_4$NP, Cr$_2$O$_3$NP, and CuONP; and these were instilled into the lungs of rats. Their immunoinflammatory potential and propensity to produce PAP were then evaluated and the underlying mechanisms were examined.

**Materials and Methods**

Nanoparticles and characterization

Metal oxide nanoparticles (NP) were purchased from commercial sources: NiONP and Co$_3$O$_4$NP (Nanostructural and Amorphous Materials, TX, USA); Cr$_2$O$_3$NP (io-li-tec, Heilbronn, Germany); CuONP (Sigma-Aldrich, Dorset, UK). The primary size and shape of NP was measured by transmission electron microscopy (JEM-1200EX II, JEOL, Tokyo, Japan). The surface areas of NP were determined by BET (Brunauer-Emmett-Teller) using a Micromeritics TriStar 3000 (Bedfordshire, UK). NP were dispersed in saline with 5% heat-inactivated rat serum as previously described [12]. Briefly, stock concentration of NP were dispersed with distilled water at 6000 cm$^2$/ml and sonicated using a probe sonicator (Philip Harris Scientific, Lichfield, UK) for 30 sec with 50% power. The NP stock (final concentration: 300 cm$^2$/ml) and heat-inactivated rat serum (final concentration: 5%) was mixed prior to addition of 0.9% physiological saline. The zeta potential of NP, a measurement of surface charge, was checked using a Zetasizer-Nano.
ZS (Malvern, Malvern Hills, UK) and hydrodynamic size and polydispersity in suspensions was analyzed using a Brookhaven 90 plus (Holtsville, NY, USA). The levels of LPS contamination in the NP suspensions were evaluated by a Limulus Amebocyte Lysate assay (Cambrex, MD, USA).

**Solubility test of NP**

To evaluate the solubility of NP in conditions mimicking the lung environment, NP at 300 cm$^2$/ml were incubated with artificial lysosomal fluid (pH 5.5) or artificial interstitial fluid (pH 7.4) for 24 h and 4 wks. Artificial lysosomal fluid and artificial interstitial fluid were prepared by formulae described previously [14, 15]. After each time point, the dissolved ions were collected by 3 rounds of centrifugation (15000 × g for 20 min) and measured by inductively coupled plasma-atomic emission spectrometry (ICP-OES; Perkin Elmer Optima 5300 DV). As a control, NP dispersed in saline at 300 cm$^2$/ml were incubated for 24 h and water soluble metal ions of the supernatant collected as described were measured using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7500ce).

**Animal experiment**

NP were dispersed in saline with 5% rat serum for intratracheal instillation into rats. Six-week old female Wistar rats (200 – 250 g) were purchased from a specific pathogen-free colony at Harlan Laboratories (Hillcrest, UK) and quarantined for 7 days prior to commencement of the study. Water and a normal diet was available ad libitum. The rats were maintained and handled under a specific license granted by the UK Home Office that ensures humane treatment and alleviation of suffering in all animal experiments. Rats were randomly assigned into groups based on body weight and NP were instilled at the same surface area dose. In our previous
studies, we tested several NP types at surface area doses of 50, 150, 250 cm²/rat and 150 cm²/rat showed a clear discrimination among NP types [1, 12]. Therefore, we chose the surface area for instillation as 150 cm²/rat and the equivalent mass doses for NiONP, Co₃O₄NP, Cr₂O₃NP, and CuONP were 163.5, 419.0, 201.6, and 515.0 µg, respectively. Ten rats were used as a vehicle control (5% rat serum in saline) whilst five rats were used for other groups. When experiments were repeated, vehicle controls were always included and so the number built up eventually reaching 10. Rats were anesthetized by isoflurane and the trachea was cannulated with a laryngoscope. Five hundred microlitres of the suspensions of NP were instilled into the lungs and the rats were sacrificed at 24 h or 4 wks post-instillation. At each time-point, rats were sacrificed by peritoneal injection of sodium pentobarbitone (200 mg/rat) and blood was taken from caudal vena cava. Bronchoalveolar lavage was obtained by cannulating the trachea with a luer port cannula (Portex, Kent, UK) connected to a syringe and the lungs were lavaged four times with 8 ml of saline. The first lavage was kept for total protein, lactate dehydrogenase (LDH), and cytokine measurements. All four lavages were pooled for cytological analysis.

**Preparation of bronchoalveolar lavage (BAL)**

Cytospin slides of the BAL were prepared as previously described [12]. Briefly, BAL was centrifuged at 250 × g for 5 min and resuspended with 1 ml of PBS. The total number of cells was counted using a nucleocounter (Chemometec, Allerod, Denmark) and 10,000 cells were attached to glass slides by cytospin at 15 × g for 5 min. The slides were fixed with 100% methanol and stained with Diff-Quik (Raymond Lamb, Eastbourne, UK). More than 500 cells in each sample were counted for differential cell count according to their morphological characteristics using a light microscope.
**Measurement of phospholipids and total proteins in the BAL**

The concentration of phospholipids in the BAL was measured using a phospholipids assay kit (Bioassay systems, CA, USA). Levels of total proteins in the BAL were measured using a bicinchoninic acid (BCA) assay (Sigma-Aldrich).

**ELISA for inflammatory mediators**

To evaluate the immunological responses, GM-CSF, MCP-1/CCL2, MIP-2/CXCL2, IL-12+p40, Th1 (IFN-γ), Th2 (IL-4), and Th17 (IL-17A) cytokines were measured using commercially available ELISA kits; MCP-1/CCL2: BD (Oxford, UK); (IL-12+p40: Invitrogen (Paisley, UK); IL-17A: eBioscience (Hatfield, UK); GM-CSF, MIP-2/CXCL2, IFN-γ, and IL-4: R&D systems (MN, USA). Cytokines in serum (1:2 dilution) and BAL (non-diluted) samples were measured according to the manufacturer’s instruction.

**Measurement of autoantibody for GM-CSF in the serum and BAL**

To measure the levels of the autoantibodies to GM-CSF, the rat GM-CSF Duoset kit from R&D systems was used. Instead of the capture antibody, serum (1:2 - 1:200 dilution) or non-diluted BAL fluids were coated on 96-well plates. After blocking with 1% BSA in PBS, GM-CSF standard (250 pg/ml) was applied to the serum or BAL coated wells for 2 h. Detection antibody, streptavidin-HRP, substrate (tetramethylbenzidine), and stop solution were respectively applied as indicated in the instruction manual.

**Immunohistochemistry for CD3, CD45RA, and Ki-67**
To evaluate the cell types infiltrated in the BAL, we performed immunohistochemistry for CD3 which is specific for mature T-cells and CD45RA which is specific for B-cells, NK cells, and naïve T-cells. Cytospin slides were hydrated in distilled water and hydrogen peroxide (3%) was applied for 15 min to quench the activity of endogenous peroxidase. Slides were blocked with normal goat serum and anti-rat CD3 antibody (AbD Serotec, Oxford, UK) or anti-rat CD45RA antibody (AbD Serotec) was applied at 1:100 dilution. Slides were washed three times and incubated for 30 min at room temperature with Envison anti-mouse IgG kit (Dako, Cambridgeshire, UK). The slides were then washed a further three times and 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories, Peterborough, UK) was applied.

To evaluate the cellular proliferation Ki-67 immunohistochemistry was performed on paraffin sections of lung tissues. Briefly, 3 µm paraffin lung sections were dewaxed and hydrated using a xylene-alcohol series. Endogenous peroxidise activity was quenched by incubating with hydrogen peroxide (3%) at room temperature for 15 min. Antigen retrieval was performed by incubating with Borg Decloaker (Biocare Medical, CA, USA) for 2 min. Anti-human Ki-67 antibody (Abcam, Cambridge, UK) was applied at 1:50 dilution. Envision anti-mouse IgG kit and DAB substrate were applied, respectively. Ki-67-positive cells in the alveolar epithelium were counted. More than 4000 cell nuclei of the alveolar epithelial cells in alveoli excluding blood vessels and bronchioles were counted under a microscopy at ×400 magnification. Percentages of Ki-67-positive cells were determined on four slides in each group and expressed as mean ± SD.

Histological analysis
One non-lavaged and four post-lavaged lungs were inflated with 10% neutral buffered formalin and fixed overnight. Lungs were then trimmed, processed, and waxed by routine histological processes. Paraffin blocks were cut into 3 µm sections and stained with H&E. Routine Periodic acid-Schiff (PAS) staining was used to measure glycoprotein accumulation. The number of lymphocyte aggregates in the alveoli excluding peribronchial lymph nodes in each lung section of all lobes was counted and multiplied by the following scale representing the diameter of aggregates: 1, less than 50 µm; 2, 50 – 200 µm; 3, bigger than 200 µm.

Transmission electron microscopy (TEM)

To evaluate the ultra-structural pulmonary changes induced by haptenic NP, we selected NiONP as a representative NP inducing DTH and PAP and instilled at 150 cm². After 4 wks, lungs of vehicle control and NiONP treatment were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer. The lung tissues were then stained en bloc with uranyl acetate, and embedded in epoxy resin. Ultra-thin (60 nm) sections were cut, stained with uranyl acetate and lead citrate, and examined with TEM (JEM-1200EX II, JEOL, Tokyo, Japan).

Test of alveolar macrophage function on clearance of DPPC

Surfactant is composed of about 90% of lipids and 10% of proteins [16]. Phospholipids and phosphatidylcholine comprises 80 – 90% of the lipids. Dipalmitoylphosphatidylcholine (DPPC) is the major component (60%) of phosphatidylcholine. The physiological levels of surfactant lipids within the lung have been estimated to be between 100 – 500 µg/ml [17]. To evaluate the effects of particle overload on the clearance of DPPC, we extracted alveolar macrophages from Wistar rats prior to culture in 24-well plates (Nunc, Roskilde, Denmark) at a density of $1 \times 10^6$
cells/ml. RPMI medium (PAA Laboratories, Somerset, UK) containing 10% fetal bovine serum, 2 mM L-glutamine (Life Technologies, Paisley, UK), 100 IU/ml penicillin, and 100 U/ml streptomycin (Life Technologies) was used. After 2 h incubation, cells were washed with pre-warmed PBS and treated with NP at 30 and 100 cm²/ml for NiONP, Co₃O₄NP, and Cr₂O₃NP, or 3 and 10 cm²/ml for CuONP for 2 h. The different dose scheme between CuONP and other NP was selected based on the cytotoxicity; CuONP showed around 10-times higher cytotoxicity than other NP. Then, NP were washed with pre-warmed PBS and synthetic DPPC (L-α-phosphatidylcholine dipalmitoyl) (Sigma-Aldrich) was applied at 250 µg/ml for a further 22 h. The turbidity of the supernatant was measured by absorbance at 600 nm. Absorbencies of DPPC treated groups were corrected by subtracting those of fresh medium and quantified by plotting a standard curve. The levels of phospholipids were measured in the serially diluted DPPC and supernatant according to the manufacturer’s instruction (Bioassay systems). Cells were also collected by brief pipetting and cytospins were prepared as described above.

Instillation of NiONP without dispersion medium

To evaluate the effects of serum coating on the surface of NP, NiONP was selected as an exemplar NP because it showed most severe toxicity in our panel of NP. Same surface area dose of NiONP without dispersion medium and therefore having big aggregates (hydrodynamic size: 6393 ± 1028 nm) was instilled into the lungs of rats which were sacrificed 4 wks after instillation. BAL fluid analysis and measurement of phospholipids and total protein in the BAL were performed using the same methods described above. Four rats were used for instillation and the data were compared with the vehicle control and well-dispersed NiONP.
Statistical analysis

Data were analyzed with GraphPad InStat Software (Version 5, GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as mean ± standard deviation (SD). To compare each treatment group, one-way analysis of variance with post hoc Tukey’s pairwise comparisons was applied. \( P < 0.05 \) was considered to be statistically significant.

Results

Physicochemical analysis of nanoparticles

Table 1 summarizes the size, surface area, hydrodynamic size, polydispersity, zeta potential, and endotoxin level of the NP panel. The size distribution measured by TEM showed that NiONP, Co\(_3\)O\(_4\)NP, and CuONP had a narrow size distribution consistent with the measure of polydispersity whilst Cr\(_2\)O\(_3\)NP showed a more heterogeneous size distribution (Table 1, Supplementary Fig. S1). All NP were well-dispersed, showed negative zeta potentials with no endotoxin found.

Solubility of nanoparticles in acid and basic condition

All NP showed minimal dissolution in the physiological saline and artificial interstitial fluid (pH 7.4) whilst solubility of NP in the artificial lysosomal fluid (pH 5.5) was heterogeneous with solubility in the order CuONP > NiONP > Co\(_3\)O\(_4\)NP > Cr\(_2\)O\(_3\)NP (Table 1, Supplementary Fig. S2). It was notable that CuONP was completely dissolved within 24 h whilst other NP showed slow and continuous dissolution.
Cytological analysis of BAL

Acute response

Figure 1 shows that, at 24 h after instillation, all NP-treated rats showed increased total cells (Fig. 1A) in the BAL but with no changes in lymphocyte numbers (Fig. 1D). The nature of the inflammation differed, however, as Cr$_2$O$_3$NP increased the numbers of macrophages (Fig. 1B) whereas NiONP, Co$_3$O$_4$NP, and CuONP increased granulocyte numbers (Fig. 1C).

Chronic response

Figure 1 shows that 4 wks after instillation animals treated with CuONP had resolved the acute neutrophilia (Fig. 1C) and were no different from the vehicle controls; rats treated with NiONP and Co$_3$O$_4$NP maintained the neutrophilia (Fig. 1C) and in addition showed increased numbers of macrophages (Fig. 1B) and lymphocytes (Fig. 1D) indicating ongoing inflammation; the inflammation induced by Cr$_2$O$_3$NP was similar to the acute response with increased macrophages only, but without evidence of lymphocyte or granulocyte recruitment. Lymphocytes in the BAL recruited by NiONP stained positively for both CD3 and CD45RA (Supplementary Fig. S3).

Pulmonary alveolar proteinosis

Blue-staining proteinaceous material dominated the background of the cytospin slides of BAL cells from NiONP and Co$_3$O$_4$NP treated rats at 4 wks and large foamy macrophages were also present (Figs. 2B and C). The BAL fluid of NiONP and Co$_3$O$_4$NP treated rats was milky and opaque whilst those from Cr$_2$O$_3$NP and CuONP treated animals were clear, similar to the vehicle control (Fig. 2F). Consistent with the appearance, the measured turbidity, levels of phospholipids and total proteins in the BAL were also significantly increased in NiONP and Co$_3$O$_4$NP but not in Cr$_2$O$_3$NP or CuONP treated animals (Figs. 2G - I).
Levels of autoantibodies for GM-CSF

As PAP can be associated with autoantibodies to GM-CSF, we tested BAL and serum samples 4 wks after instillation, and no autoantibodies were detected (data not shown).

Effects of nanoparticles on ability of macrophages (AM) to catabolise DPPC

When added to AM, large amounts of NP were phagocytosed within 2 h (Fig. 3C No DPPC) although CuONP could not be seen at the magnification used. When the culture medium was then removed, the cells washed and fresh medium supplemented with DPPC (250 µg/ml) was added, NiONP and Co$_3$O$_4$NP treated AM became foamy, whilst Cr$_2$O$_3$NP and CuONP maintained a normal macrophage appearance and size (Fig. 3C DPPC). Without DPPC, foamy macrophages were not seen in any treatment groups. In concert with the development of a foamy appearance, the AM exposed to NiONP and Co$_3$O$_4$NP were unable to clear DPPC from the medium (Fig. 3A). Thus the appearance of foamy macrophages indicated a build-up of DPPC within the macrophages and was indicative of their failure to process and clear DPPC. There was a clear contrast between the substantial clearance of DPPC from the medium of the control, Cr$_2$O$_3$NP and CuONP treated AM and the much reduced capacity for their clearance shown by AM treated with the NiONP and Co$_3$O$_4$NP (Figs. 3A and B).

Histological analysis

Acute response

Consistent with the BAL data, at 24h after instillation, NiONP induced moderate perivascular and peribronchiolar inflammation and mild neutrophil infiltration into the alveoli
Supplementary Fig. S4; Co3O4NP did not induce any interstitial inflammation but there was mild neutrophilia in the alveoli; Cr2O3NP treated rats showed no inflammation in any compartment; CuONP treated rats had moderate neutrophilic inflammation both in the interstitium and alveoli.

Chronic response

Four wks after instillation, NiONP and Co3O4NP exposed lungs showed foamy macrophages with proteinaceous deposits in the alveoli (Figs. 4B and C) and large interstitial lymphoid aggregates (Fig. 4F). The lymphocytes aggregates score was not variable between post-lavaged and non-lavaged lung (Fig. 4F). The proteinaceous materials stained positively for glycoprotein with PAS staining (Supplementary Fig. S5). Despite the increased numbers of macrophages seen in BAL, Cr2O3NP exposed lungs showed no inflammatory reaction in the interstitium (Fig. 4D). CuONP treated rats showed mild to moderate granulomatous inflammation (Fig. 4E).

**Ki-67 immunohistochemistry**

Figure 5 shows that lung tissues at 4 wks after instillation of NiONP and Co3O4NP showed a substantial population of cells with Ki-67-positive nuclei which anatomically and morphologically appeared to be type II alveolar epithelial cells. We based our identification of type II cells as the proliferating cells of (1) Ki-67 positivity, (2) their anatomical position where septa wall, (3) it is well established that the only proliferating cell in alveolar wall during lung injury is the type II alveolar epithelial cell [18]. Lungs from rats exposed to the vehicle control, Cr2O3NP, and CuONP showed very few Ki-67 positive nuclei in the alveolar region (Figs. 5D and E). The percentage of Ki-67 positive cells in the alveolar walls in NiONP and Co3O4NP
treated lungs was around 20% whilst vehicle control, Cr$_2$O$_3$NP, CuONP showed around 3% positivity (Fig. 5F).

**TEM analysis of NiONP exposed lungs**

Control lungs showed normal alveolar septae, capillaries, type II cells and occasional alveolar macrophages in the alveolar lumen (Figs. 6A and B). In contrast, numerous foamy macrophages and profuse lipo-proteinaceous material were found in the alveolar spaces in NiONP-treated lungs 4 wks after instillation (Figs. 6C and D). Foamy macrophages contained numerous vesicles and lamellar bodies, which are known to contain surfactant protein and phospholipids. In addition, NiONP particles were visible inside the foamy macrophages (Fig. 6E). Alveolar type II cells were increased in number and contained numerous vacuoles compared to vehicle control (Fig. 6F). The proteinaceous materials in the alveoli were consistent with surfactant in the form of large-aggregates and lamellar bodies.

**Cytokines in the BAL**

To evaluate the mediators that might account for the observed inflammatory responses, we analysed BAL and serum for the chemokines MCP-1/CCL2 (chemotactic for monocytes, granulocytes, and lymphocytes), MIP-2/CXCL2 (chemotactic for granulocytes and lymphocytes), IL-12+p40 (induces Th1 effector cells), the pro-inflammatory Th1 associated cytokine IFN-$\gamma$, the Th2 associated cytokine IL-4, the Th17 effector cell associated cytokine IL-17A , and for GM-CSF (induced by inflammation and by lung exposure to NP).

In the serum samples from NP-exposed rats, there were no significant changes in any cytokines compared to vehicle control at either time-point, whereas in BAL the different NP treatments
induced different patterns of cytokine/chemokine release indicating that the inflammatory responses generated were compartmentalised in the lungs.

Figure 7 shows that levels of MCP-1/CCL2 were significantly increased by NiONP and Co$_3$O$_4$NP at both the acute and chronic phases whilst CuONP induced MCP-1/CCL2 only in the acute phase. The levels of MIP-2/CXCL2 were significantly increased by NiONP, Co$_3$O$_4$NP, and CuONP 24 h post-instillation and had returned to control levels by 4 weeks. The levels of IL-12+p40 were significantly increased by CuONP at 24 h and by NiONP and Co$_3$O$_4$NP 4 wks after instillation. The levels of IFN-$\gamma$ were significantly elevated compared to vehicle control only by NiONP at 4 wks. IL-17A was significantly increased at 24 h by CuONP and at 4 wks by NiONP instillation compared to vehicle control. GM-CSF and IL-4 showed no significant changes in any treatment group compared to controls.

The effects of serum coating on the toxicity of NiONP

Both serum coated NiONP (NiO$_{\text{serum}}$) and NiONP without corona (NiO$_{\text{uncoated}}$) produced DTH-like inflammation and PAP (Supplementary Figs. S6 and S7). However, NiO$_{\text{uncoated}}$ showed significantly less potential for producing DTH-like inflammation (number of lymphocytes) and PAP (levels of phospholipids and total protein) compared to NiO$_{\text{serum}}$.

Discussion

In our previous work, we found that metal oxide nanoparticles (NP) release water-soluble ions and that instillation of these into rat lungs induced contrasting types and kinetics of inflammation [1, 12]. Some metal ions including Ni(II), Co(II, III), Cr(III), and Cu(II) have been reported to
induce DTH (characterised by Th1 and/or Th17 effector cell activation) in human skin via haptenisation of proteins [8]. Since such metal ions induce allergic responses in skin and metal oxide NP release metal ions, we hypothesized that NP comprised of the oxides of haptenic metals might induce DTH-like inflammation and pathology in the lungs.

The pathological appearance of lungs at 4 wks largely agreed with the BAL cellular profile. Cr$_2$O$_3$NP produced no inflammatory effects acutely or chronically whilst CuONP caused a transient acute inflammation in the BAL that had resolved leaving a modest granulomatous inflammation evident in histological sections by 4 wks. In contrast both NiONP and Co$_3$O$_4$NP produced acute neutrophilic inflammation and caused worsening DTH-like mononuclear inflammation, characterized by PAP, epithelial proliferation, lymphocytic foci, and granulocytic infiltration of the airspace. NiONP has been known to cause acute neutrophilic inflammation [1, 12] and chronic inflammation consisting of lymphocytes and neutrophils [12, 19]. Co$_3$O$_4$NP, without dispersion media, did not significantly increased the PMN number when instilled at surface area dose of 250 cm$^2$/rat although increased reactive oxygen species were detectable which might trigger acute neutrophilic inflammation [1]. This discrepancy with our data might be due to the improved dispersion of our NP; well-dispersed NP shows much higher inflammatory potential compared to poorly-dispersed NP [20]. CuONP cause acute neutrophilic inflammation and chronic granulomatous inflammation when instilled into lungs of rats [12, 21]. However, the chronic effects of Co$_3$O$_4$NP and Cr$_2$O$_3$NP and the immunological effects of our panel of NP have not been previously reported.

Allogeneic rat serum acts as an excellent dispersant for NP without risks of ‘foreignness’. When NP are inhaled, they deposit on the surfactant lipoprotein surface lining of the lung. This surfactant lipoprotein immediately forms a corona on the surface of the NP. Both serum-coated
NiONP (NiO_{serum}) and NiONP without corona (NiO_{uncoated}) showed significant DTH-like inflammation and PAP compared to vehicle control. However, the potential for provoking these pathologies by NiO_{serum} were significantly greater than NiO_{uncoated}. This difference is most likely due to the improved dispersion rather than serum protein-specific effects in the NiO_{serum}. All NP showed minimal solubility in the physiological saline and basic artificial interstitial fluid (pH 7.4) which mimics pulmonary interstitial fluid [15] whilst NP showed heterogeneous range of solubilities in acidic condition (pH 5.5) which mimics the lysosomal environment of alveolar macrophages [14, 22]. In this acid environment, CuONP were completely dissolved within 24 h whilst other NP showed a slow and continuous dissolution pattern in order of NiONP > Co_3O_4NP > Cr_2O_3NP. Therefore, because of fast dissolution and clearance, CuONP might have no opportunity to be haptenic [23]. On the other hand, NiONP and Co_3O_4NP showed slow continuous dissolution providing a ‘slow-release’ of challenging hapten over 4 wks, eventually provoking DTH-like inflammation. Cr_2O_3NP showed the least and minimal dissolution among our panel of NP and this might not be sufficient to be haptenic. This dissolution is consistent with the findings of many particles in alveolar macrophages treated with NiONP, Co_3O_4NP, and Cr_2O_3NP whilst no CuONP could be seen 4 wks after instillation. The kinetics of micron-sized nickel compounds showed that the half-life of the inhaled soluble form of nickel compound (NiSO_4) was 2-3 days in rats [24] and less than 1 day in mice [24] whilst an insoluble form of nickel compound (NiO, 2-2.4 µm) was 33 days [24] or 120 days [25] in rats. The half-life of the inhaled nano-sized NiO (139 ± 12 nm) was 62 days in rats [26]. In this study we have not challenged putative antigen at a distal site, since this was not the aim of the study, therefore we have not demonstrated classical DTH. However, we contend that the ongoing release of soluble ions from biopersistent NP resident in the lungs provides ongoing challenge producing a DTH-
like response. Neither the primary particle size nor hydrodynamic size showed any relationship with the DTH potential.

The mass doses of the panel of NP used here (163.5 – 515.0 µg) can be contextualised to plausible lung dose. For example a predicted alveolar retained mass of 163.5 µg would result from 10 days of exposure to a 650 µg/m$^3$ cloud of NP with aerodynamic diameter of 100 nm (consistent with hydrodynamic size of this study) according to the multiple-path particle dosimetry deposition model (MPPD model) [27]. According to the US EPA, the ACGIH TLV (American Conference of Governmental and Industrial Hygienists’ threshold limit value) of insoluble nickel and metal compounds is 200 µg/m$^3$ and 1500 µg/m$^3$, respectively [28].

The cellular inflammatory responses we measured in the lungs had a biologically plausible relationship with the cytokines/chemokines measured in the BAL. Initially CD3-positive T lymphocytes were a feature of the BAL infiltrate in NiONP and Co$_3$O$_4$NP instilled lungs. None of the NP tested induced IL-4 secretion, indicating a lack of any Th2-related immunity. MCP-1/CCL2 plays an important role in recruiting monocytes/macrophages [29], neutrophils [30], and T-lymphocytes [31] and MIP-2/CXCL2 recruits neutrophils [32]. Therefore, the increased levels of MCP-1/CCL2 and MIP-2/CXCL2 induced by NiONP, Co$_3$O$_4$NP, and CuONP during the acute phase were consistent with the increased number of granulocytes in BAL. The increases in MCP-1/CCL2 induced by NiONP and Co$_3$O$_4$NP during the chronic phase were also consistent with the increased number and type of BAL cells. MCP-1/CCL2 might mediate DTH by stimulating Th1-related cytokines expression [29]. IL-12 promotes Th1 responses and stimulates IFN-$\gamma$ production [33]. IL-17A is associated with Th17 effector cells and neutrophil recruitment and also stimulates Th1 immunity [34]. NiONP and Co$_3$O$_4$NP induced a sustained increase in lymphocyte and macrophage cell numbers in BAL and interstitial lymphocytic aggregates.
However, NiONP and Co₃O₄NP had a different cytokine induction profile with NiONP inducing secretion of both Th1 (IL-12+P40 and IFN-γ) and Th17 (IL-17A) associated cytokines in BAL at the later time-point, whereas Co₃O₄NP instilled animals only had measurable IL-12+P40 indicating less Th1 and no sustained Th17 activation. In contrast, despite releasing metal ions known to cause skin DTH [8], Cr₂O₃NP and CuONP showed no lymphocytic response in BAL or the lung interstitium and no sustained Th1 or Th17 related cytokine response. There is overwhelming evidence in the literature that the 4 metals used in NP oxide form in this study are haptenic in the skin as metal ions [8]. We show here that, in oxide nanoparticulate form, the same metals are heterogeneous in terms of their ability to induce pulmonary DTH-like responses in rats. NiONP and Co₃O₄NP stood out in contrast to the other two metal oxide NP as causing sustained lymphocytic infiltrate into the BAL, lymphocyte aggregates in lung section and increases in both MCP1/CCL2 and IL-12+P40 in the BAL. In addition, NiONP caused increases in IFN-γ and IL-17A. Thus classical markers of TH1/DTH responses are increased, albeit with the NiONP appearing to be more potent than Co₃O₄NP in this regard.

The PAP induced by NiONP and Co₃O₄NP in rats has similar features to human and primate alveolar lipoproteinosis [35]. Surfactant lipoprotein forming the surfactant epithelial lining fluid is normally in dynamic equilibrium through the actions of the alveolar type II cells which synthesize and secrete it and alveolar macrophages and alveolar type II cells which clear and recycle it [11]. PAP is either genetic or secondary to haematological malignancies, autoimmune diseases, or particle inhalation and can be associated with production of anti-GM-CSF autoantibodies [10]. Particles can produce PAP by dust overload or with a toxic dust at lower dose. Particles known to induce secondary alveolar proteinosis include silica, titanium, indium-tin oxide, aluminium, and cement [10]. However, the pathogenesis of PAP induced by particle
inhalation is poorly understood. Deposition of particles in the lung induces alveolar type II cell proliferation and/or impairs the function of surfactant clearance by killing alveolar macrophages [36]. However, a recent study with indium-tin oxide [37] and a large cohort study from Japan [38] both showed that particle or dust exposure might be linked with autoimmune alveolar proteinosis by inducing autoantibodies to GM-CSF. We found no evidence of anti-GM-CSF autoantibodies in any of the animals with PAP. Therefore, at least in our experiments, PAP induced by NiONP and Co$_3$O$_4$NP were not associated with autoantibodies against GM-CSF. In the present study, we found that NiONP and Co$_3$O$_4$NP induced PAP. In vitro these NP impaired the clearance of DPPC by primary alveolar macrophages and induced foamy macrophage morphology, mimicking events in the lungs. In contrast Cr$_2$O$_3$NP and CuONP did not cause foamy macrophage formation and did not affect DPPC clearance by macrophages in vitro although the NP were fully engulfed. NiONP and Co$_3$O$_4$NP treated rats also demonstrated increased proliferation of alveolar type II cells, which are responsible for surfactant production. Thus the over-production of surfactant by proliferation of alveolar type II cells and the impairment of macrophage surfactant clearance probably combined to induce the PAP in NP-exposed lungs.

In the present study, we only focused on a small sub-set of metal oxide NP and their long-term immunological effects following a single instillation. We do not suggest that these effects are nano-specific and in fact many kinds of micron-sized particles cause immunological effects e.g. Beryllium. It is important to discover the mechanism of NP effects whatever they are, since NP exposures are set to increase as NP are utilised more in industrial processes, necessitating hazard identification to aid the risk assessment process. Neither do we consider that the mechanism shown here for the two immunologically-active NP is generic for all NP. Different NP cause
their effects by different mechanisms dependent on their composition and shape and we focus here only on one small sub-set of NP type and one type of immuno-pathological response. Nanosized particles have been known to cause more severe pulmonary toxicity than micron-sized particles [19], and the immuno-pathological responses that are elicited might be much greater than micron-sized particles as a result of greater retention and increased solubility from their greater surface area. This is also supported by increased DTH potential of well-dispersed NiONP (92 nm hydrodynamic size) than highly-aggregated NiONP (6 µm hydrodynamic size). The National Toxicology Program (NTP) in USA performed a 2-year inhalation study using nickel compounds and showed that micron-sized nickel compounds, including an insoluble form (NiO-2.2 ± 2.6 mm) and soluble forms (NiSO₄ and Ni₃S₂) both caused alveolar proteinosis in the lung and lymphoid hyperplasia in the bronchial lymph nodes [39-41]. Because the NP studied here are being manufactured for diverse purposes including catalysts, semiconductors and pigments [42], accidental occupational exposure is likely. Our data suggest that a single accidental exposure to certain haptenic metal oxide NP might produce prolonged and worsening immunopathological responses.

In conclusion, NP comprised of oxides of metals known to be haptenic in skin had variable potential to induce DTH-like responses and PAP in the lungs of rats after a single instillation exposure. Whilst NiONP and Co₃O₄NP exposures were associated with a worsening immuno-inflammatory response producing PAP and immunologically-mediated lung damage, Cr₂O₃NP and CuONP were not active in this regard. This has important implications for risk management of some NP indicating that a single elevated exposure to some NP in an occupational setting might trigger a progressive lung disease characteristic of delayed type hypersensitivity. This may
be a result of the slow release of metal ions from NP causing repeated cycles of haptenisation of host proteins leading to chronic auto-immune inflammation.

Acknowledgements

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References


**Figure Legends**

Figure 1. Differential cell counts of the BAL fluid at 24 h or 4 wks after instillation of metal oxide nanoparticles at 150 cm$^2$. (A), number of total cells; (B), number of macrophages; (C), number of total granulocytes; (D), number of lymphocytes. Each treatment group was compared with vehicle control (VEH) for statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 8$ for all vehicle control and $n = 4$ for other treatment groups.
Figure 2. Bronchoalveolar lavage analysis 4 wks after instillation of NiONP, Co$_3$O$_4$NP, Cr$_2$O$_3$NP, and CuONP at 150 cm$^2$. Representative images of BAL cells of vehicle control (A), NiONP (B), Co$_3$O$_4$NP (C), Cr$_2$O$_3$NP (D), and CuONP (E). Lymphocytes (arrow) were recruited
by NiONP and Co$_3$O$_4$NP but not by Cr$_2$O$_3$NP or CuONP. Neutrophil (arrowhead) infiltration was seen in NiONP and occasionally seen in the Co$_3$O$_4$NP and CuONP treatment groups. Diff-Quik staining. Bar scale = 50 µm. (F), representative colour of BAL fluid at 4 wks after instillation of metal oxide NP at 150 cm$^2$. Four samples per group were pooled in quartz cuvette and pictures were taken by a digital camera. (G), turbidity was measured at the absorbance of 600 nm. Turbidity (G), levels of phospholipids (H) and total proteins (I) in the BAL were significantly increased by NiONP and Co$_3$O$_4$NP instillation. Each treatment group was compared with vehicle control (VEH) for statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n = 8 for all vehicle control and n = 4 for other treatment groups.

Figure 3. Clearance of DPPC by primary cultured alveolar macrophages (AM). DPPC levels were measured by absorbance at 600 nm and quantified by a standard curve ($R^2$=0.999). NiONP,
Co$_3$O$_4$NP, and Cr$_2$O$_3$NP were added at 30 and 100 cm$^2$/ml whilst CuONP was added at 3 and 10 cm$^2$/ml. NP were treated for 2 h and cultured with or without DPPC (250 µg/ml). The levels of phospholipids in the DPPC and cell supernatant were measured by commercially available kits. The levels of cleared DPPC (A) and phospholipids (B) by AM were significantly inhibited by NiONP and Co$_3$O$_4$NP whilst Cr$_2$O$_3$NP and CuONP were comparable to vehicle control. Each treatment group was compared with vehicle control for statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 8$ for all vehicle control and $n = 4$ for other treatment groups. (C), treatment of DPPC to NiONP and Co$_3$O$_4$NP treated groups made AM foamy whilst Cr$_2$O$_3$NP and CuONP did not. Without DPPC, any NP did not induce foamy AM. The representative cytospin images were taken from low dose groups (3 cm$^2$/ml for CuONP and 30 cm$^2$/ml for others). Diff-Quik staining. Bar = 25 µm.

Figure 4. Lung histology 4 wks after instillation of metal oxide nanoparticles to rats at 150 cm$^2$. Representative lung histology of vehicle control (A), NiONP (B), Co$_3$O$_4$NP (C), Cr$_2$O$_3$NP (D), and CuONP (E). NiONP and Co$_3$O$_4$NP produced lymphocyte aggregates (arrow), foamy
macrophages, and deposition of proteinaceous material in the alveoli. Cr$_2$O$_3$NP did not cause any inflammatory reactions whilst CuONP induced chronic granulomatous inflammation (arrowhead). Tissues were stained with H&E. Bars = 100 µm. (F), lymphocyte aggregates score was determined by counting the number of lymphocyte aggregates in the alveoli excluding peribronchial lymph nodes in each lobe and multiplied by the following scale representing the diameter of aggregates: 1, less than 50 µm; 2, 50 – 200 µm; 3, bigger than 200 µm. Each treatment group was compared with vehicle control for statistical significance: $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$.

Figure 5. Immunohistochemistry for Ki-67 in the lungs 4 wks after instillation of metal oxide nanoparticles at 150 cm$^2$. Ki-67-positive cells were found in the alveolar type II-like cells (arrow) in the NiONP (B) and Co$_3$O$_4$NP (C) treated lungs. Vehicle control (A), Cr$_2$O$_3$NP (D) and CuONP (E) showed no positive signals in the alveolar epithelium. Each panel is a representative section of the lungs from the various exposed groups. Bars = 50 µm. (F), percentages of Ki-67-positive nuclei in the alveolar region excluding blood vessels and bronchioles were determined
by four slides for each group and expressed as mean ± SD. Each treatment group was compared with vehicle control for statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 8$ for all vehicle control and $n = 4$ for other treatment groups.

Figure 6. Representative TEM images of vehicle control (A and B) and NiONP (C - F) 4 wks post-instillation to rats. (A), normal alveoli structure consisted with thin type I cells (black arrow). Compared to macrophage (black arrow) in vehicle control (B), foamy macrophages (black arrow) were found in the alveoli of NiONP treated lung (C). Note that foamy macrophages contained numerous complex phospholipoprotein inclusions. (C), proteinous materials (white arrow) were deposited in the alveoli and higher power view (D) revealed those materials were consistent with surfactant large-aggregate forms (white arrow) and lamellar bodies (black arrow). (E), the higher magnification rectangle in (B) revealed NiONP (black arrow) to be present inside foamy macrophages. (F), proliferating and vacuolated type II epithelial cells (white arrow) in NiONP treated lungs.
Figure 7. Concentrations of MCP-1/CCL2 (A), MIP-2/CXCL2 (B), IL-12+p40 (C), IL-17A (D), IFN-γ (E), and IL-4 (F) in the BAL after instillation of metal oxide NP at 150 cm². Each treatment group was compared with vehicle control for statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 8$ for all vehicle control and $n = 4$ for other treatment groups.
Table 1. Physicochemical analysis of metal oxide nanoparticles.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>NiONP</th>
<th>Co$_2$O$_4$NP</th>
<th>Cr$_2$O$_3$NP</th>
<th>CuONP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary size$^a$</td>
<td>5.3 ± 1.9</td>
<td>18.4 ± 5.0</td>
<td>205 ± 129</td>
<td>23.1 ± 7.2</td>
</tr>
<tr>
<td>Hydrodynamic size (nm)$^b$</td>
<td>92.50 ± 4.10</td>
<td>185.2 ± 31.3</td>
<td>136.2 ± 60.2</td>
<td>112.1 ± 17.3</td>
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<tr>
<td>Polydispersity$^b$</td>
<td>0.21 ± 0.07</td>
<td>0.10 ± 0.02</td>
<td>0.68 ± 0.04</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>S.A. (m$^2$/g)$^c$</td>
<td>91.8</td>
<td>35.8</td>
<td>74.42</td>
<td>29</td>
</tr>
<tr>
<td>Zeta potential (mV)$^d$</td>
<td>-21.0 ± 5.0</td>
<td>-21.6 ± 0.7</td>
<td>-28.3 ± 6.0</td>
<td>-25.3 ± 4.4</td>
</tr>
<tr>
<td>Endotoxin$^e$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mass (µg) per animal</td>
<td>163.5</td>
<td>419.0</td>
<td>201.6</td>
<td>515.0</td>
</tr>
<tr>
<td>Metal ions (ppm)$^f$ – 24 h</td>
<td>Ni(II)</td>
<td>Co(II, III)</td>
<td>Cr(III)</td>
<td>Cu(II)</td>
</tr>
<tr>
<td>Saline</td>
<td>4.00 (1.56)$^g$</td>
<td>0.09 (0.02)</td>
<td>1.70 (0.62)</td>
<td>5.00 (0.61)</td>
</tr>
<tr>
<td>Basic (pH 7.4)</td>
<td>3.97 (1.16)</td>
<td>0.08 (0.01)</td>
<td>4.34 (1.58)</td>
<td>5.38 (0.65)</td>
</tr>
<tr>
<td>Acid (pH 5.5)</td>
<td>22.9 (8.93)</td>
<td>9.74 (1.58)</td>
<td>4.05 (1.47)</td>
<td>804 (97.3)</td>
</tr>
<tr>
<td>Metal ions (ppm) – 4 wks</td>
<td>Ni(II)</td>
<td>Co(II, III)</td>
<td>Cr(III)</td>
<td>Cu(II)</td>
</tr>
<tr>
<td>Basic (pH 7.4)</td>
<td>6.47 (2.52)</td>
<td>0.07 (0.01)</td>
<td>0.00 (0.00)</td>
<td>7.98 (0.97)</td>
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<tr>
<td>Acid (pH 5.5)</td>
<td>87.6 (34.1)</td>
<td>51.3 (8.34)</td>
<td>3.50 (1.27)</td>
<td>832 (100)</td>
</tr>
</tbody>
</table>

$^a$Primary size of NP was measured by transmission electron microscopy.
$^b$Hydrodynamic size and polydispersity of NP dispersed with 5% rat serum in saline was determined using a Brookhaven 90 plus (Holtsville, NY, USA).
$^c$Surface area (S.A.) was measured with Micromeritics TriStar 3000 (Bedfordshire, UK).
$^d$Zeta potential of NP dispersed with 5% rat serum in saline was measured using a Zetasizer Nano ZS (Malvern, Malvern Hills, UK).
$^e$Endotoxin levels were measured by a Limulus Amebocyte Lysate assay (Cambrex, MD, USA) and detection limit was 10 pg/ml.
$^f$Water soluble metal ions of NP in saline, artificial interstitial fluid (basic), or artificial lysosomal fluid (acid) were analysed by ICP-MS (Agilent 7500ce) or ICP-OES (Perkin Elmer Optima 5300 DV ICP-OES).
$^g$Parenthesis represent the percentage of dissolution compared to initial mass.
ND = not detectable.