



Mechanistic hazard differentiation of cerium oxide and barium sulfate nanoparticles using a human-relevant lung model

Isidora Lončarević^a, Seyran Mutlu^{b,c}, Roman Mmenda Fortunatus^a, Nathalie Jung^a, Jorge Larios^{a,d}, Sandor Balog^{a,d}, Martina Dzepic^{b,c}, Alke Petri-Fink^a, Fabian Blank^{b,c}, Barbara Rothen-Rutishauser^{a,*}

^a Adolphe Merkle Institute, University of Fribourg, Chemin des Verdiers 4, Fribourg 1700, Switzerland

^b Lung Precision Medicine (LPM), Department for BioMedical Research (DBMR), University of Bern, Switzerland

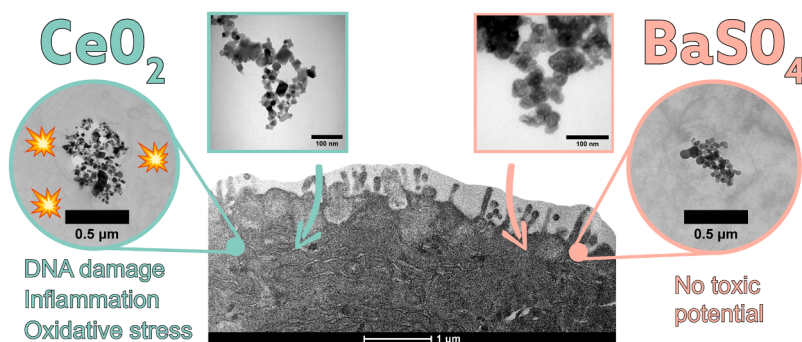
^c Department for Pulmonary Medicine, Allergy and Clinical Immunology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland

^d National Center of Competence in Research Bio-Inspired Materials, University of Fribourg, Chemin des Verdiers 4, Fribourg 1700, Switzerland

HIGHLIGHTS

- CeO₂ and BaSO₄ nanoparticles show distinct hazard profiles in a human-relevant Calu-3/MDM co-culture model.
- CeO₂ induces inflammatory signaling and transcriptional responses consistent with oxidative stress and DNA damage.
- BaSO₄ shows minimal biological activity consistent with *in vivo* and human data.
- Molecular endpoints detect early stress signals before epithelial barrier disruption.
- AOP173 framework enables translational comparison with rodent inhalation and human studies.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Cerium oxide
Barium sulfate
Pulmonary fibrosis
Nanotoxicology
Occupational exposure

ABSTRACT

Increasing regulatory pressure to reduce animal testing has accelerated the development of New Approach Methodologies (NAMs) for hazard assessment. This study employed a Calu-3/monocyte-derived macrophages (MDMs) co-culture at the air-liquid interface to assess the biological effects of cerium oxide (CeO₂) and barium sulfate (BaSO₄) nanoparticles, two occupationally relevant materials. Doses ranged from 6 to 324 μg/cm² for BaSO₄ and 9 to 324 μg/cm² for CeO₂, and samples were analyzed on days 1, 3, and 7. Epithelial barrier integrity, cytotoxicity, inflammatory cytokine secretion, and targeted transcriptional responses were mapped onto the adverse outcome pathway for inflammation-induced pulmonary fibrosis (AOP173) and compared with published rodent inhalation and human data. CeO₂ induced sustained basolateral interleukin-8 secretion with NF-κB activation and upregulation of oxidative stress, DNA damage, and proteotoxic stress markers peaking at day 3 at the middle dose (108 μg/cm²). Epithelial barrier integrity remained intact, while molecular endpoints detected early AOP-relevant stress responses. BaSO₄ elicited minimal activity across all endpoints, consistent with *in vivo* and human data, and was correctly identified as a low-hazard material. By integrating multi-

* Corresponding author.

E-mail address: barbara.rothen@unifr.ch (B. Rothen-Rutishauser).

<https://doi.org/10.1016/j.jhazmat.2026.143515>

Received 12 July 2026; Received in revised form 20 August 2026; Accepted 3 September 2026

Available online 5 September 2026

0304-3894/© 2026 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

endpoint responses within an AOP framework, this study demonstrates that human cell-based co-culture models can mechanistically discriminate nanoparticles with distinct hazard profiles.

1. Introduction

Nanoparticles (NPs) are increasingly used in industrial, commercial, and consumer applications, raising concerns about occupational and environmental exposure, particularly via inhalation, the primary route of uptake for airborne particulates [1]. Among these materials, cerium oxide (CeO₂) and barium sulfate (BaSO₄) represent two widely produced NPs with contrasting physico-chemical and toxicological profiles, making them valuable reference materials for hazard assessment.

CeO₂ NPs are used as diesel fuel additives to improve combustion efficiency and reduce soot emissions, as polishing agents in the semiconductor and optics industries, and as electrolyte materials in solid oxide fuel cells [2]. Their combustion-related use results in direct release of CeO₂ NPs into the ambient environment, contributing to airborne nanoparticle loads in both urban and occupational settings and raising concerns about inhalation exposure across the general population and workforce alike. CeO₂ is biopersistent in the lung, with impaired clearance and overload observed in rodent studies at subchronic exposure levels [3] and its accumulation has been linked to rare earth pneumoconiosis in occupationally exposed workers, including optical lens grinders and photoengravers [4–6]. Notably, no nano-specific occupational exposure limit has been established by the Occupational Safety and Health Administration (OSHA) for CeO₂; it currently falls under the general category of Particulates Not Otherwise Regulated (PNOR), with a permissible exposure limit (PEL) of 5 mg/m³ for the respirable fraction [7]. This regulatory gap, combined with the growing evidence of CeO₂'s pulmonary toxicity and its environmental release as a combustion byproduct, underscores the need for mechanistic hazard data from human-relevant test systems.

In contrast, BaSO₄ is widely used in paints, coatings, and plastics, as well as in medical imaging as a radiopaque contrast agent. It is generally regarded as low-toxicity, with OSHA classifying it separately from soluble barium compounds and assigning a comparatively low PEL of 10 mg/m³ (total) and 5 mg/m³ (respirable fraction) [7]. In occupational settings, prolonged inhalation of BaSO₄ dust can cause baritosis, a benign, non-fibrotic pneumoconiosis characterized by striking radiographic opacities, no impairment of lung function or symptoms, and gradual recovery after exposure [8,9].

Beyond differences in use and regulatory status, the contrasting pulmonary behavior of CeO₂ and BaSO₄ is also due to different dissolution behavior in biological fluids. Continuous-flow dissolution studies under simulated lysosomal conditions classify BaSO₄ as a partially dissolving material whose ion release is governed by local ion saturation rather than particle size, with a predicted dissolution half-time (~11 days) that closely matches its measured *in vivo* pulmonary clearance half-time (9.6 days) [10]. CeO₂, in contrast, undergoes comparatively little dissolution, consistent with the prolonged retention and overload potential described above [3]. This divergence has been observed directly at the cellular level: in a human alveolar epithelial monoculture model, both NPs were predominantly retained in the apical compartment with minimal basal translocation, yet the intracellular fraction of BaSO₄ declined markedly faster between 24 and 80 h than that of CeO₂, a difference attributed to BaSO₄'s higher dissolution rate under lysosomal-mimicking conditions [11]. These findings suggest that the two NPs follow different mechanisms upon internalization, which underline their contrasting hazard profiles.

The contrasting hazard profiles of CeO₂ and BaSO₄ have also been established through a series of *in vivo* inhalation studies. In a comprehensive short-term inhalation screening of 14 NPs, CeO₂ ranked among the materials with higher toxic potency and BaSO₄ among those with the lowest, based on bronchoalveolar lavage parameters and histopathology

[12]. Moreover, both materials were selected as representative NPs within the European NANoREG program and the OECD sponsorship framework for nanomaterial testing and were further evaluated in the EU Horizon 2020 PATROLS project, which developed guidance for realistic *in vitro* dosing of engineered nanomaterials [13].

Despite extensive *in vivo* characterization, translating these findings into a mechanistic understanding using human-relevant *in vitro* models remains challenging. A range of *in vitro* models, including monocultures, co-cultures, and advanced three-dimensional airway tissues, have been used. One study demonstrated that rat alveolar macrophages could distinguish biologically active from passive NPs, including CeO₂ and BaSO₄, with good correlation to short-term *in vivo* data, yet this approach relies on a single cell type, and therefore its applicability is limited to materials that are phagocytosable and well dispersed, capturing only macrophage-mediated mechanisms without accounting for direct epithelial effects, epithelial-immune cell crosstalk, or barrier function [14]. Moreover, across different studies, NPs are applied under submerged, air–liquid interface (ALI) or pseudo-ALI (sometimes referred to as quasi-ALI) conditions [11,15]. Notably, ALI exposures have consistently demonstrated greater sensitivity and improved concordance with *in vivo* outcomes than traditional submerged approaches [16–18]. Pseudo-ALI exposure falls between the levels of ALI (i.e. aerosol exposure) and submerged cultures. This method is technically accessible and easier to reproduce across laboratories; therefore, it is widely used. It has been shown to generate biological responses comparable to those obtained by aerosol-based ALI delivery [19,20], supporting the validity of this approach for assessing the nanoparticle hazard under physiologically relevant conditions without the need for specialized aerosol-generation equipment.

A structured framework for interpreting mechanistic *in vitro* data is provided by the adverse outcome pathway (AOP) concept [21], a sequence of causally linked biological events that connect a molecular initiating event to an adverse outcome relevant to regulatory decision-making. Mapping *in vitro* endpoints onto AOP key events allows hazard testing to move beyond descriptive endpoints toward mechanistic interpretation grounded in *in vivo* relevance. However, in practice, *in vitro* studies differ in model complexity, exposure conditions, and the number and types of biological endpoints, thereby limiting cross-comparison and mechanistic interpretation. Although advanced models offer clear advantages by incorporating multiple cell types, polarized epithelial architecture, and physiologically relevant exposure, their multi-endpoint responses are rarely mapped onto AOP, particularly for materials such as CeO₂ and BaSO₄, because the relevant inhalation AOPs remain largely under development with qualitative key event relationships, so these readouts are typically reported as hazard profiles rather than positioned as key events along a causal pathway [21].

The AOP framework has gained increasing traction as a tool for regulatory decision-making. The successful regulatory adoption of AOP-based *in vitro* test guidelines for skin sensitization, including OECD Test Guidelines 442 C, 442D, and 442E, integrated within the defined approaches framework of OECD TG 497, demonstrates the practical translational potential of the AOP framework for replacing animal testing [22–25], and similar efforts are now underway for inhalation toxicity endpoints [26]. AOP173, which describes the pathway from substance interaction with lung-resident cell membranes to inflammation-induced pulmonary fibrosis, provides a structured framework for mapping *in vitro* endpoints onto causally linked key events and evaluating whether a given test system captures the relevant biological processes [27].

In this study, we used a human co-culture model composed of Calu-3 cells (bronchial epithelial cell line) and monocyte-derived macrophages

(MDMs), cultured at the air-liquid interface, to investigate the biological effects of CeO₂ and BaSO₄ NPs across a wide dose range. This model was originally optimized for long-term, repeated aerosol exposure at the air-liquid interface [28], and subsequently shown to be transferable and reproducible across seven independent laboratories within the EU Horizon 2020 PATROLS project [29]. More recently, the exposure protocol and endpoint selection were further refined and directly benchmarked against *in vivo* outcomes using crystalline silica (DQ12) as a reference particle, demonstrating the model's ability to capture biologically meaningful, time-resolved inflammatory responses [30]. Building on this established model, we assessed particle physicochemical behavior in cell culture media, epithelial barrier integrity, cytotoxicity, inflammatory cytokine secretion, and targeted gene expression spanning oxidative stress, inflammation, DNA damage, and apoptosis pathways. By mapping these endpoints onto the key events of AOP173 and comparing them with published *in vivo* data, we aimed to determine whether CeO₂ and BaSO₄ can be mechanistically differentiated using this human-relevant *in vitro* model, and to identify the early key events linking their distinct physicochemical behavior to inflammation-induced pulmonary fibrosis. These findings contribute to a more mechanistic, material-specific characterization of inhaled nanoparticle hazard, while also supporting the development of alternative testing strategies that reduce reliance on animal models.

2. Materials and methods

2.1. Chemicals and reagents

All the chemical reagents were purchased from Sigma-Aldrich (Switzerland), while all cell culture reagents were purchased from Thermo Fisher Scientific (Switzerland) unless stated otherwise.

2.2. Test nanoparticles

Cerium oxide (CeO₂) NM-212 and barium sulfate (BaSO₄) NM-220 nanoparticles (NPs) were obtained from Fraunhofer IME and used within the OECD Working Party on Manufactured Nanomaterials Testing Program [31] as selected representative materials. Full physicochemical characterization data for the materials used in this study are publicly available in the PATROLS project deliverable [32].

2.3. Nanoparticle dispersion protocol

NPs were dispersed according to the Nanogenotox protocol [33] for toxicity testing in 5 mL of endotoxin-free water (Cat ref. 1897399), without bovine serum albumin (BSA). Briefly, 145.8 mg of the material powder was weighed into glass vials (20 mL, N24, flat-bottom; MACHEREY-NAGEL, Düren, Germany; Cat. No. 702021), pre-wetted with 100 µL ethanol, and mixed with 5 mL of endotoxin-free water to a stock concentration of 29.16 mg/mL. The solution was sonicated using a Branson SFX-550 sonifier model equipped with a model 102-C converter (Emerson, Baar, Switzerland) to reach a total power of 7056 J (7.35 W for 21 min). During sonication, the glass vial was kept on ice to prevent excessive heating of the samples.

Final exposure concentrations were prepared by serial dilution of the stock in cell culture medium to achieve doses ranging from 6 to 324 µg/cm² for BaSO₄ and 9–324 µg/cm² for CeO₂ on the 0.9 cm² inserts. For example, the highest dose of 324 µg/cm² corresponds to 9.72 mg/mL in 30 µL applied per insert. All suspensions were always prepared on the day of cell exposure and vortexed right before exposure (Vortex-Genie 2, Scientific Industries, USA).

2.4. Nanoparticle morphology and primary size characterization by transmission electron microscopy

To image the NPs, transmission electron microscopy (TEM) was

performed using an FEI Technai Spirit (FEI, Hillsboro, OR, USA) operating at 120 kV. Ten microliters of the sample were drop-cast onto a carbon film on a copper 300-square-mesh grid (Electron Microscopy Sciences, Pennsylvania, PA, USA) and dried at room temperature before visualizing the NPs with a 2048 × 2048-pixel wide-angle Veleta CCD camera (Olympus, Tokyo, Japan). Image processing, including scale bar inclusion, contrast adjustments, and particle analysis, was performed using Fiji (ImageJ version 1.54). TEM images were used to determine particle size. Using FLJI (ImageJ), individual, distinguishable NPs were manually outlined using the polygon selection tool to obtain projected particle areas. The equivalent circular diameter (ECD), defined as the diameter of a circle with the same projected area, was calculated as

$$EDC = 2\sqrt{\left(\frac{A}{\pi}\right)}. \text{ A total of 160 BaSO}_4 \text{ and 210 CeO}_2 \text{ NPs were analyzed.}$$

2.5. Colloidal stability and hydrodynamic size assessment

The hydrodynamic diameter was determined by dynamic light scattering (DLS) using an Anton Paar Litesizer 500 particle analyzer (Anton Paar, Graz, Austria) operating with a 658 nm laser and a scattering angle of 175°.

For the size measurement series, measurements were performed using disposable cuvettes in backscatter detection mode at a target temperature of 25 °C, following a 30 s equilibration time. Data were analyzed using a narrow distribution model with an advanced cumulant algorithm. Measurements were conducted in manual mode, with a single run per sample and a 30 s measurement duration. Automatic filter and focus settings were applied. Water was used as the dispersant, with a refractive index of 1.3303 and a viscosity of 0.0008903 Pa·s. Stock suspensions were diluted with Milli-Q® water (pH = 6.0) or Calu-3 cell culture medium (pH = 7.2) to a final concentration of 90 µg/mL for measurement. This corresponds to a dilution factor of 3–100 × relative to the concentrations used in cell exposure experiments, ensuring that particle concentrations were suitable for reliable DLS measurements without multiple scattering or detector saturation.

For the Zeta Potential Series 10 measurements, an Omega cuvette (Mat. No. 225288) was used at a target temperature of 25 °C, following a 30 s equilibration time. Data were analyzed using the Smoluchowski approximation with a Henry factor of 1.5. Power adjustment was conducted in automatic mode with a maximum voltage of 200 V. Measurements were acquired in manual mode with up to 100 runs per sample. Water was used as the dispersant, with a refractive index of 1.3303, viscosity of 0.0008903 Pa·s. For the measurements, NPs were diluted in Milli-Q® water (pH = 6.0) and cell culture media (pH = 7.2). After incubation of 1 h in cell culture media, NPs were centrifuged, then resuspended in Milli-Q® water, centrifuged again, and resuspended in Milli-Q® water before being measured. For the data plotting, GraphPad Prism Version 10.2.3 was used.

2.6. Cell-free radical scavenging activity assessment

The antioxidant activity of NPs was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, adapted from Basnet et al. with minor modifications [34]. Briefly, 0.5 mL of an ethanolic DPPH solution (60 µM) was mixed with 0.5 mL of the sample. Cerium oxide (CeO₂) NPs were prepared in Milli-Q® water and complete cell culture media (CCM), consistent with exposure conditions used in biological experiments. Corresponding blanks (sample with ethanol replacing DPPH) and controls (DPPH with ethanol replacing the sample) were included. Additionally, CCM alone was measured, and its contribution was subtracted from the sample readings.

The reaction mixtures were incubated for 30 min at room temperature in the dark, after which absorbance was measured at 520 nm using a V-670 spectrophotometer (Jasco Corporation, Tokyo, Japan). Samples were diluted when necessary to ensure measurements fell within the

linear range of the Beer–Lambert law. For cerium-containing samples, appropriate dilutions were applied ($15 \times$ in CCM and $25 \times$ in Milli-Q® water), while barium-containing samples were analyzed without dilution. Antioxidant activity was expressed as the percentage of DPPH radical scavenging relative to the control.

For the data plotting and statistics, GraphPad Prism Version 10.2.3 was used.

2.7. Cell lines and primary cell culture conditions

At every step during cell culture and exposure, cells were kept in an incubator under controlled conditions (humidified atmosphere, 37°C , 5% CO_2). Cell numbers were determined using an EVE™ bench-top automated cell counter (Witex AG, Switzerland) with the trypan blue exclusion method (0.4% trypan blue solution in phosphate buffer saline (PBS) (Cat. No. 10010–015).

The human bronchial epithelial cells (Calu-3) were purchased from the American Type Culture Collection (ATCC, Rockville, USA). Calu-3 cells were cultured in complete cell culture media (CCM) composed of MEM + GlutaMAX (Cat. No. 41090–028) supplemented with heat inactivated 10% fetal bovine serum FBS (Cat. No. A5256701), 1% Pen Strep (Cat. No. 15140–122), 2% Amphotericin B (Cat. No. 15290–026) and 1% Non-Essential Amino Acids (NEAA) solution (Cat. No. 11140–035). Heat inactivation was done by incubating the pre-thawed FBS bottle for 30 min at 56°C in a water bath with occasional shaking. The cells were seeded at a density of 90,000 cells/ cm^2 Falcon® 12-well PET permeable supports (0.4 μm pore size, 0.9 cm^2 growth area; Corning, Corning, NY, USA; Cat. No. 353180) placed in a companion 12-well flat-bottom plate with a low-evaporation lid (Corning Life Sciences, Corning, NY, USA, Cat. No. 353043). The cells were kept in the incubator, and the medium was renewed every 2–3 days.

Human MDMs were differentiated from monocytes isolated from buffy coats provided by the Swiss Transfusion Center (Bern, Switzerland), as described previously [35,36]. In brief, isolated blood monocytes were cultured in 6-well cell culture plates (Corning, FALCON®, United States) for six days at a density of 10^6 cells/mL in 3 mL Roswell Park Memorial Institute (RPMI)-1640 (Cat. No. 42401–018) cell culture medium supplemented with 10% (v/v) heat inactivated FBS, 1% L-glutamine (Cat. No. 25030–024), and 1% Pen Strep. For MDMs differentiation, 10 ng/mL of macrophage colony-stimulating factor (M-CSF; Miltenyi 130–096–485) was added to the cell culture medium for 6 days. Experiments involving primary monocyte isolation from human blood were approved by the committee of the Federal Office for Public Health Switzerland (reference number: 611–1, Meldung A110635/2) for the Adolphe Merkle Institute.

2.8. Establishment of the Calu-3/MDMs air–liquid interface co-culture model

The cell culture model is well-established and was used in a recent interlaboratory comparison study [29]. More details on the Calu-3 model can be found elsewhere in the literature [36–38]. In brief, Calu-3 cells were cultured under submerged conditions for 7 days. After 7 days, the apical medium was removed, and the cells were cultured at the ALI for an additional 7 days. On day 14, after differentiation into MDMs, cells were scraped off the wells, counted, and added to the Calu-3 monolayer to obtain a co-culture. As not all MDMs adhere to the Calu-3 cells, 39,000 MDMs were added in a volume of 0.2 mL to achieve a final concentration of 25,000 MDMs/ cm^2 [28]. After 4 h, the apical medium was removed, and the co-culture was returned to the incubator. Monocytes were derived from different donors for each biological repetition.

2.9. Nanoparticle exposure protocol and dose selection

Exposure of the cell cultures with NPs was performed under pseudo-ALI conditions according to the already developed PATROLS Guidance

Document 3016 [39] without washing the apical side of the insert and adjusting the volume to the surface of the 12-well insert. In brief, 30 μL of the previously prepared particle solution was added to the apical side of the co-culture insert with a surface area of 0.9 cm^2 . Based on the known volume of particle suspension and surface area of the inserts, a calculation was made, and the concentrations were reported in $\mu\text{g}/\text{cm}^2$. The plate was returned to the incubator for the desired exposure period of 24 h, 3 days, or 7 days. During exposures, only the basal medium was refreshed every 2–3 days, while the apical compartment was left undisturbed, and the apically applied NPs remained *in situ* throughout the exposure period.

2.10. Endotoxin contamination screening

Both CCM and NPs suspension media aliquots were analyzed for endotoxin contamination using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific, Cat. No. A39552) according to the manufacturer's instructions. For all samples, endotoxin levels were below the 1 EU/mL threshold considered acceptable for cell-based experiments (CCM = 0.193 EU/mL; CeO_2 = 0.130 EU/mL; BaSO_4 = 0.417 EU/mL).

2.11. Mycoplasma contamination monitoring

A mycoplasma test was performed to detect potential Mycoplasma infections in cell culture. Two mL of cell supernatant from a cell culture flask, which has been in contact with cells for at least 24 h, was collected for mycoplasma detection. MycoAlert™ Assay was performed according to the manufacturer's user guide Lonza protocol (Catalog #: LT07–318). For Calu-3 cells, the gain-to-ratio (210) was 0.275, below the acceptable limit of 0.9.

2.12. Plastic embedding and ultrathin sectioning

After the experiment, the co-cultures on the PET inserts were washed 3 times with PBS, and the membrane was cut out with a scalpel. Cells were then rinsed with PBS and fixed for 18 h at 4°C in 2.5% glutaraldehyde (EMS, Cat. No. 16216), 4% paraformaldehyde (EMS, Cat. No. 15714) prepared in 0.1 M cacodylate buffer (EMS, Cat. No. 11652). Following fixation, samples were washed five times for 5 min each in 0.1 M cacodylate buffer. Post-fixation was carried out using 1% osmium tetroxide (EMS, Cat. No. 19150), 1.5% potassium hexacyanoferrate (II) trihydrate (Sigma-Aldrich, Cat. No. 455989) in 0.1 M cacodylate buffer for 1 h at room temperature, followed by six washes for 5 min each in 0.1 M cacodylate buffer.

Samples were subsequently dehydrated through a graded ethanol series consisting of 25%, 50%, 70%, 80%, 90%, and 100% ethanol (Sigma-Aldrich, Cat. No. 1.00983.1000), followed by 100% anhydrous ethanol, with each step performed for 15 min at room temperature. Dehydration was completed with two incubations in anhydrous acetone (Sigma-Aldrich, Cat. No. 1.00014), each lasting 20 min at room temperature.

Resin infiltration was performed by sequential incubation in 25%, 50%, and 75% epoxy resin mixtures diluted in anhydrous acetone for 1 h per step, followed by overnight incubation in 100% epoxy resin mixture. The epoxy resin formulation consisted of 46.1% (w/w) dodecenyl succinic anhydride (Sigma-Aldrich, Cat. No. 45346), 29.0% (w/w) Durcupan ACM Component A (Sigma-Aldrich, Cat. No. 44611), 22.3% (w/w) Epon 812 substitute (Sigma-Aldrich, Cat. No. 45345), and 2.7% (w/w) dibutyl phthalate (Sigma-Aldrich, Cat. No. 524980). Subsequently, samples were incubated for 2 h at room temperature in epoxy resin supplemented with 2.25% (v/v) DMP-30 accelerator (Sigma-Aldrich, Cat. No. 45348). The resin was then refreshed with the same DMP-30-containing mixture prior to polymerization at 60°C for 28 h.

Ultrathin sections (50–70 nm) were prepared using a 45° Ultra diamond knife (Diatome, Cat. No. 20-US) mounted on a Reichert Ultracut E

ultramicrotome. Sections were collected onto formvar-carbon-coated copper grids (Agar, Cat. No. AGS162–5). Samples were examined using a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope operated at an accelerating voltage of 120 kV and equipped with a side-mounted Veleta CCD camera (Olympus). The micrograph images were processed with ImageJ 1.54p (Fiji, NIH, USA; Java 1.8.0_322, 64-bit).

2.13. Epithelial barrier integrity assessment

At 24 h after exposure, 500 μ L cell culture medium was added to the apical side of the cells, and the epithelial barrier integrity was evaluated by transepithelial electrical resistance (TEER) measurements using a Millicell® ERS-2 (Electrical Resistance System, Millipore, Switzerland) equipped with an STX01 electrode (World Precision Instruments, Switzerland). All TEER measurements were recorded in the presence of a 500 μ L cell culture medium at 37 °C in the apical compartment of the inserts and a 1500 μ L medium in the basolateral compartment. Measurements were performed inside a biosafety cabinet at room temperature, with cultures being outside the incubator for 25 min. Before each TEER recording, the electrodes were sterilized in 70% ethanol, rinsed with pre-warmed sterile 1x HBSS (Cat. No. 14025–092) and briefly equilibrated in pre-warmed cell culture medium. This procedure was done before measuring TEER in each insert. A blank insert (without cells) was measured to determine offset values, which were then subtracted from the sample readings. Measurements were performed in three technical replicates (each insert was measured 3 times, with the probe positioned at different areas of the insert) and 3 biological replicates.

2.14. Cytotoxicity assessment

The release of LDH into the supernatant was assessed using an LDH cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany, Ref. 11644793001), according to the manufacturer's protocol. Each sample was tested in a single experiment, with three independent replicates. LDH activity was quantified photometrically by measuring at 490 nm (reference wavelength at 630 nm). For positive controls, cell cultures were exposed apically to 2% Triton X-100 in PBS for 30 min, and the negative control was untreated co-cultures. Samples collected from both apical and basal sides were centrifuged at 14,000 $\times$ g for 5 min to ensure that all debris is removed from the supernatant and then tested.

2.15. Quantification of inflammatory mediators

Because Calu-3 cells form a polarized epithelium, cytokine secretion occurs in a directional manner; therefore, both the apical and basolateral compartments were analyzed to capture the full inflammatory profile of the co-culture. At 1 day, 3 days, or 7 days after exposure, 500 μ L cell culture medium was added to the apical side of the inserts. After 30 min, the apical and basolateral supernatants were collected separately and stored at –80 °C for cytokine and chemokine analysis. IL-8, IL-6, and TNF- α were selected because they are established markers of the key inflammatory event in AOP173 and enable direct comparison with cytokine panels reported in the *in vivo* rodent inhalation literature for cross-species alignment (see Discussion, Table 1). Release of inflammatory cytokines interleukin IL-6, IL-8, and tumor necrosis factor- α (TNF α) was measured in the supernatants using ELISA kits (Biotechne, R&D systems, Minneapolis, USA, IL-6 Cat. No. DY206, IL-8 Cat. No. DY208, and TNF α Cat. No. DY210). Each sample was tested in a single experiment, with three independent replicates. Cytokine analysis was performed according to the manufacturer's protocol. For ELISA, absorbance was measured at 450 nm with a reference wavelength of 570 nm.

2.16. Total RNA extraction and quality control

After collecting the supernatant for the ELISA assay, the cells were carefully washed 3 times with PBS, and the total RNA was isolated from the co-culture. Cell lysis was performed directly on the insert, using 100 μ L of BL + TG buffer (Promega Madison, WI, USA, Ref. Z6011), and total RNA was extracted using ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA, Ref. Z6011) following the technical manual from the manufacturer. The quantity and quality of RNA were assessed by Thermo Scientific™ NanoDrop™ 2000 Spectrophotometer. The 260/280 and 260/230 absorbance ratios were evaluated, and only RNA samples with ratios of $\sim$ 2.0 (typically 1.9–2.2) were taken forward for cDNA synthesis and RT-qPCR analysis.

2.17. RNA reverse transcription and gene expression analysis by real-time-qPCR

The reverse transcriptase reaction was performed with the Omniscript RT system (Qiagen, Hilden, Germany, Cat. No. 205113), OligodT (Microsynth, Balgach, Switzerland, Cat. No. 35 and 36), and RNasin Plus RNase Inhibitor (Promega, Madison, WI, USA, Switzerland, Cat. No. NZ2611), described previously [40]. In brief, the synthesis of complementary DNA (cDNA) was performed by using 6.5 μ L of isolated RNA (250 ng), 1 μ L oligo-dT primer (10 μ M), 0.25 μ L RNase inhibitor, 1 μ L dNTP Mix (5 mM), 0.25 μ L Omniscript reverse transcriptase (1 Unit), and 1 μ L buffer RT.

Real-time PCR was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Reactions were carried out in a final volume of 10 μ L by mixing 2 μ L 5-fold diluted cDNA with 5 μ L SYBR-green master mix (Fast SYBR Green master mix, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. 4385617), 2 μ L nuclease-free water (Promega, Madison, WI, USA, Cat. No. P119A), and 2 μ L primer mix (91 nM).

Thermal cycling conditions consisted of an initial denaturation at 95 °C for 20 s, followed by 40 amplification cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. A melt curve analysis was performed at the end of each run, consisting of a preconditioning step at 95 °C for 15 s, followed by incubation at 60 °C for 1 min, and a continuous temperature ramp from 60 °C to 95 °C, with fluorescence acquisition to verify amplification specificity.

Relative expression levels were calculated as fold change compared to untreated co-cultures with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*) as internal standard genes. These reference genes were selected based on empirical assessment of expression stability, as their Ct values showed minimal variation (CV <3%) across all experimental conditions and treatments. Primers were purchased from Thermo Fisher Scientific (Zug, Switzerland). Details about the primers are included in [Supplementary Table 1](#).

2.18. RNA reverse transcription and gene expression analysis by qPCR array

The panel was designed to investigate the molecular key events most consistently implicated in metal-oxide nanoparticle lung toxicity, with oxidative stress as the core mode of action and the expected point of divergence between redox-active CeO₂ and the passive BaSO₄ nanoparticle, as introduced above. Genes were selected through a targeted literature review of markers reported in published *in vivo* rodent inhalation studies, allowing direct cross-species benchmarking of our *in vitro* findings without generating additional animal data (Table 1). It spanned four functional categories: oxidative stress, DNA damage/repair, inflammation, and growth/signaling, with NF- κ B signaling (*RELA*) linking redox imbalance to the inflammatory key events central to AOP173. The full panel composition is provided in [Supplementary Table 2](#).

Total RNA (800 ng) was reverse transcribed using the QuantiNova Reverse Transcription Kit (Qiagen, Hilden, Germany; Cat. No. 205411) according to the manufacturer's instructions. Briefly, RNA was combined with the gDNA Removal Mix (2 μ L) and internal control (1 μ L) in a final volume of 15 μ L with RNase-free water, and incubated at 45 °C for 2 min followed by a pause at 25 °C to eliminate genomic DNA contamination. Reverse transcription components, reverse transcriptase (1 μ L) and Transcription Mix (4 μ L), were then added on ice directly to the 15 μ L gDNA elimination reaction, yielding a final reaction volume of 20 μ L. Reverse transcription was carried out with the following thermal cycling conditions: annealing at 25 °C for 3 min, reverse transcription at 45 °C for 10 min, and reaction inactivation at 85 °C for 5 min. The resulting cDNA was diluted by the addition of 90 μ L RNase-free water (final volume 110 μ L) prior to use.

Gene expression profiling was performed using custom QuantiNova LNA PCR Flexible Panels (Qiagen, Hilden, Germany; Cat. No. 249960, Variant No. 96W-24_12–23; custom panel SBFA89194) in combination with the QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany; Cat. No. 208056), on a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each panel reaction was prepared by combining 100 μ L of diluted cDNA with 260 μ L of SYBR Green Master Mix, 2.6 μ L of ROX reference dye, and 157.4 μ L of RNase-free water (total volume: 520 μ L), and distributing the mixture across the 96-well panel plate. Thermal cycling was performed under the following conditions: reverse transcriptase activation at 50 °C for 10 min, initial PCR activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s and combined annealing/extension at 60 °C for 10 s, with fluorescence data collected at each extension step. A melting curve analysis was performed at the end of each run to confirm amplification specificity.

Each panel included two dedicated controls: a Human Genomic DNA Control (HGDC) to verify gDNA removal efficiency and an RNA Quality Internal Control (QIC) to detect the synthetic RNA spike-in added during the reverse transcription step. A Positive PCR Control (PPC) well confirmed PCR amplification performance. The panel profiled 18 target genes involved in oxidative stress response, heat shock response, apoptosis, and xenobiotic metabolism: *NQO1*, *GCLC*, *GSR*, *TXNRD1*, *HSPA1A*, *LPO*, *NOX1*, *CYBB*, *RELA*, *BCL2*, *BAX*, *EGFR*, *HSP90AA1*, *ATM*, *CYP1A1*, *AHR*, *CDKN1A*, and *GADD45A*. Gene expression was normalized using three reference genes, *GAPDH*, *RPLP0*, and *YWHAZ*, selected for their stable expression across experimental conditions. Relative gene expression was calculated using the $\Delta\Delta C_q$ method, with statistical analysis and visualization performed in R. Five genes (*BCL2*, *LPO*, *NOX1*, *CYBB*, *CYP1A1*) with a mean Ct > 30 across all conditions were excluded due to low or undetectable expression in the Calu-3/MDM co-culture system, resulting in a final panel of 13 genes used for statistical analysis and visualization.

2.19. Statistical analysis

All experiments were performed with $n = 3$ biological replicates; for TEER, three technical replicates per insert were averaged prior to analysis. Data are presented as mean $\pm$ standard deviation unless otherwise stated, and a threshold of $p < 0.05$ was considered statistically significant (* $p < 0.05$, ** $p < 0.01$).

TEER and LDH data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. Cytokine data (IL-8, IL-6, TNF- α) were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test, comparing each treated group with the untreated control of the corresponding day; Dunnett's test was used for treatment-versus-control comparisons and Tukey's test for pairwise comparisons across conditions. TEER, LDH, and cytokine analyses and plots were performed in GraphPad Prism v10.2.3.

For the transcriptional panel, statistical comparisons were performed on normalized Ct values (ΔC_t) rather than fold-changes. Each gene was analyzed separately at each timepoint, with each treated dose compared

against the material-matched untreated control (CeO₂ doses vs untreated CeO₂-plate controls; BaSO₄ doses vs untreated BaSO₄-plate controls; $n = 3$ biological replicates). Normality was assessed per gene and timepoint; normally distributed data were analyzed by one-way ANOVA with Dunnett's post-hoc comparison against the untreated control, and non-normally distributed data by the Kruskal–Wallis test with Dunn's post-hoc test. P-values were adjusted for multiple comparisons across the panel using the Benjamini–Hochberg (false discovery rate) method. No comparison reached statistical significance before or after correction; the transcriptional data are therefore reported as fold-change trends. Analyses and visualization were performed in R v4.5.0 (RStudio 2026.01.1 +403) using the dplyr, tidyr, purrr, broom, and ggplot2 packages.

3. Results

3.1. Particle characterization

To investigate the morphology and size of nanoparticles (NPs), transmission electron microscopy (TEM) was used. Cerium oxide (CeO₂) NPs appeared angular and faceted, displaying irregular polyhedral morphologies (Fig. 1A). In contrast, barium sulfate (BaSO₄) NPs exhibited a more rounded morphology (Fig. 1B). For both materials, TEM imaging in Milli-Q water revealed aggregates composed of irregular particles. CeO₂ NPs were polydisperse with an equivalent circular diameter (ECD) of 20.06 ± 10.84 nm, while BaSO₄ NPs showed an ECD of 30.65 ± 12.34 nm, as calculated from the TEM images of 210 and 160 particles, respectively (Supplementary Figure 1).

Zeta potential measurements were conducted for both materials in water and complete cell culture medium (CCM) (Fig. 1C). CeO₂ NPs exhibited a zeta potential of +14.32 mV in water and –27.58 mV in CCM, whereas BaSO₄ NPs showed values of –3.14 mV in water and –25.97 mV in CCM.

To further assess the colloidal stability of the NPs in CCM, DLS measurements were performed. Both materials formed heterogeneous dispersions with aggregates present (Supplementary Figure 2).

3.2. Antioxidative capacity

The antioxidative capacity of the NPs themselves in both Milli-Q® water and CCM was determined using a cell-free DPPH radical scavenging assay, which reflects surface redox reactivity. CCM itself showed intrinsic antioxidant background activity (~15%), which was accounted for in the interpretation. CeO₂ showed concentration-dependent scavenging activity in Milli-Q® water, reaching a plateau at higher concentrations. After subtracting the CCM background, CeO₂ showed meaningful scavenging activity only above approximately 8 mg/mL (~267 μ g/cm² under the exposure conditions used here) in CCM. BaSO₄ showed no scavenging activity beyond CCM background levels despite increasing concentration (Supplementary Figure 3).

3.3. Uptake of nanoparticles by cells

To confirm intracellular particle uptake, TEM imaging was performed at the highest dose for both materials on days 1 and 7. For CeO₂, NPs were taken up predominantly by MDMs (cells on top of the monolayer), where they accumulated in large membrane-bound vesicles, and this pattern was observed at both day 1 and day 7 (Fig. 2). Calu-3 cells also internalized CeO₂ NPs, but at substantially lower levels, with NPs confined to small discrete vesicles (Supplementary Figure 4).

For BaSO₄, intracellular NPs were detected in Calu-3 cells at day 7, but at markedly lower levels than CeO₂, with only a few NPs visible across multiple regions of interest (Supplementary Figure 5).

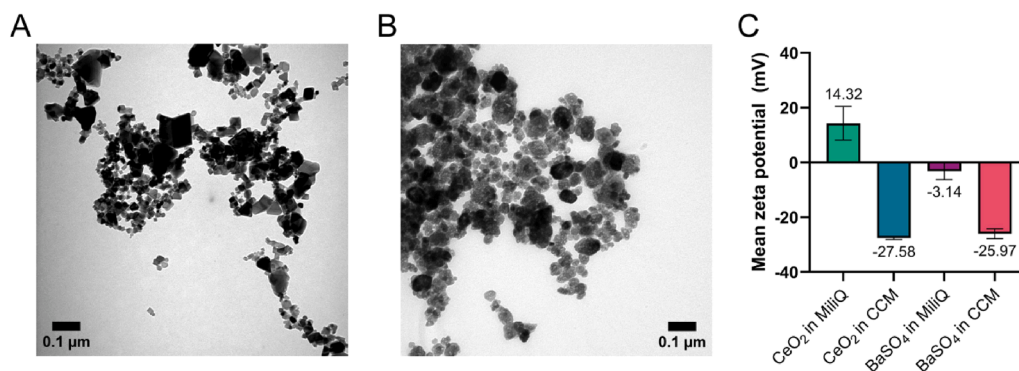


Fig. 1. Physicochemical characterization of CeO₂ and BaSO₄ nanoparticles. (A–B) TEM images of CeO₂ and BaSO₄ NPs showing their distinct morphologies. (C) Zeta potential measured in water and cell culture medium (CCM).

3.4. Cytotoxicity and membrane integrity

To confirm whether nanoparticle exposure affects epithelial barrier integrity, TEER was measured after 1, 3, and 7 days of exposure to a range of concentrations (9, 54, 108, 219, 324 µg/cm² for CeO₂ and 6, 18, 54, 162, 324 µg/cm² for BaSO₄). TEER remained largely stable across all concentrations and time points, with average values of 343.47 ± 15.15 Ω·cm² for CeO₂ and 354.27 ± 16.01 Ω·cm² for BaSO₄ (Fig. 3A,B; two-way ANOVA with Tukey's multiple comparisons test). Minor but statistically significant decreases over time were observed at 108 µg/cm² CeO₂ between day 3 and day 7 ($p = 0.0075$), though absolute TEER values remained well within the range expected for an intact epithelial barrier in this model. This was further supported by unchanged *OCN-1* expression across all conditions (Supplementary Figure 6). Moreover, TEM imaging confirmed the ultrastructure of the junctional complex, including tight junctions, adherens junctions, and desmosomes, at the highest doses of both NPs on Day 1 and Day 7 (Fig. 4).

To test the adverse effects of NPs on co-cultures, LDH was measured after 1, 3, and 7 days of exposure. CeO₂ exposure induced a slight, dose-dependent elevation in LDH release with the increase of concentration, which was not statistically significant (two-way ANOVA with Tukey's multiple comparisons test, $p > 0.05$). A slight increase over time was observed at 216 µg/cm² between day 1 and day 7 ($p = 0.0433$), though absolute LDH values remained low across all conditions (Fig. 3C). In contrast, BaSO₄ did not induce LDH release, indicating no detectable cytotoxicity at any tested concentration (Fig. 3D).

3.5. Inflammatory cytokine secretion

Cytokine secretion was measured in both apical and basolateral compartments for interleukin-8 (IL-8), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) at low (CeO₂: 9 µg/cm²; BaSO₄: 6 µg/cm²), middle (CeO₂: 108 µg/cm²; BaSO₄: 54 µg/cm²), and high (CeO₂: 324 µg/cm²; BaSO₄: 324 µg/cm²) doses over 1, 3, and 7 days.

Overall, IL-8 concentrations were consistently higher in the apical compartment (up to ~10,000 pg/mL) compared to the basal compartment (up to ~4000 pg/mL) (Fig. 5A,B). However, there was a significant increase in IL-8 secretion on the basolateral side (Fig. 5B) at all doses upon CeO₂ exposure, with the most pronounced effect observed at the middle dose ($p = 0.002$ on day 1, $p = 0.006$ on day 3, and $p = 0.002$ on day 7). In contrast, BaSO₄ did not induce significant IL-8 release to either side (Fig. 5E,F), although a trend toward decreasing basal IL-8 with increasing concentration was observed.

IL-6 levels remained near baseline on both sides for both materials, although slightly higher values were observed on the apical side (Fig. 5C,D,G,H).

TNF-α was below the detection limit at all doses and time points (Supplementary Figure 7).

3.6. Transcriptional responses of Calu-3/MDMs co-cultures

To gain mechanistic insight beyond secreted cytokines, we examined transcriptional changes across a targeted panel of 13 genes spanning oxidative stress, DNA damage/repair, inflammation, and growth/signaling.

CeO₂ induced a transient upregulation of oxidative stress defense genes (*TXNRD1*, *GSR*), stress response/proteostasis markers (*HSPA1A*, *HSP90*), inflammatory/NF-κB regulator (*RELA*), and DNA damage/repair genes (*ATM*, *GADD45A*) at day 3, particularly at the middle dose (108 µg/cm²). Mean fold-changes were highest for *HSPA1A* (2.4) and *RELA* (2.1), with more modest increases in *TXNRD1* (1.9) and *ATM* (1.8); none reached statistical significance at any dose or timepoint after correction for multiple comparisons. By day 7, most genes across all pathways were downregulated to below baseline levels (Fig. 6A).

In contrast, BaSO₄ elicited minimal transcriptional changes. Non-significant upregulation of *HSPA1A*, *HSP90*, and *RELA* was detected at day 3, at the middle dose (54 µg/cm²), as well as slight upregulation of *HSPA1A* at day 7 for the lowest dose (6 µg/cm²) (Fig. 6B).

Additionally, qPCR analysis was performed to further investigate NF-κB signaling and activation of the p53 pathway, including genes related to oxidative stress (*SOD2*, *HMOX1*) and inflammation (*TGF-β*, *TNF-α*, *IL-1β*, *IL-6*, *IL-8*). None of these genes showed significant up- or down-regulation over 7 days (Supplementary Figure 8 A,B).

4. Discussion

Inhalation of nanoparticles (NPs) such as cerium oxide (CeO₂) and barium sulfate (BaSO₄) is a growing occupational concern, yet mechanistic hazard data from human-relevant test systems remain limited. In this study, we employed a Calu-3/MDMs co-culture to assess whether material-specific biological responses can be captured *in vitro* across a dose range anchored to *in vivo* exposures.

4.1. Cell model selection

The Calu-3/MDMs co-culture was selected because it combines a human-relevant, polarized epithelial barrier with immune cells, capturing both epithelial- and macrophage-driven mechanisms of particle-induced injury in one system rather than relying on a single cell type, and because its transferability and reproducibility have already been established across independent laboratories [28,29]. This choice was further informed by our previous work using DQ12 crystalline silica as a reference particle in the same model, where a standard 24-hour exposure produced no measurable inflammatory response, but extending post-exposure assessment to 3 and 7 days, aligned with the *in vivo* sampling timeline, revealed a delayed yet reproducible cytokine response that correlated with *in vivo* inflammatory endpoints [30]. We therefore applied the same time-resolved exposure design (days 1, 3, and

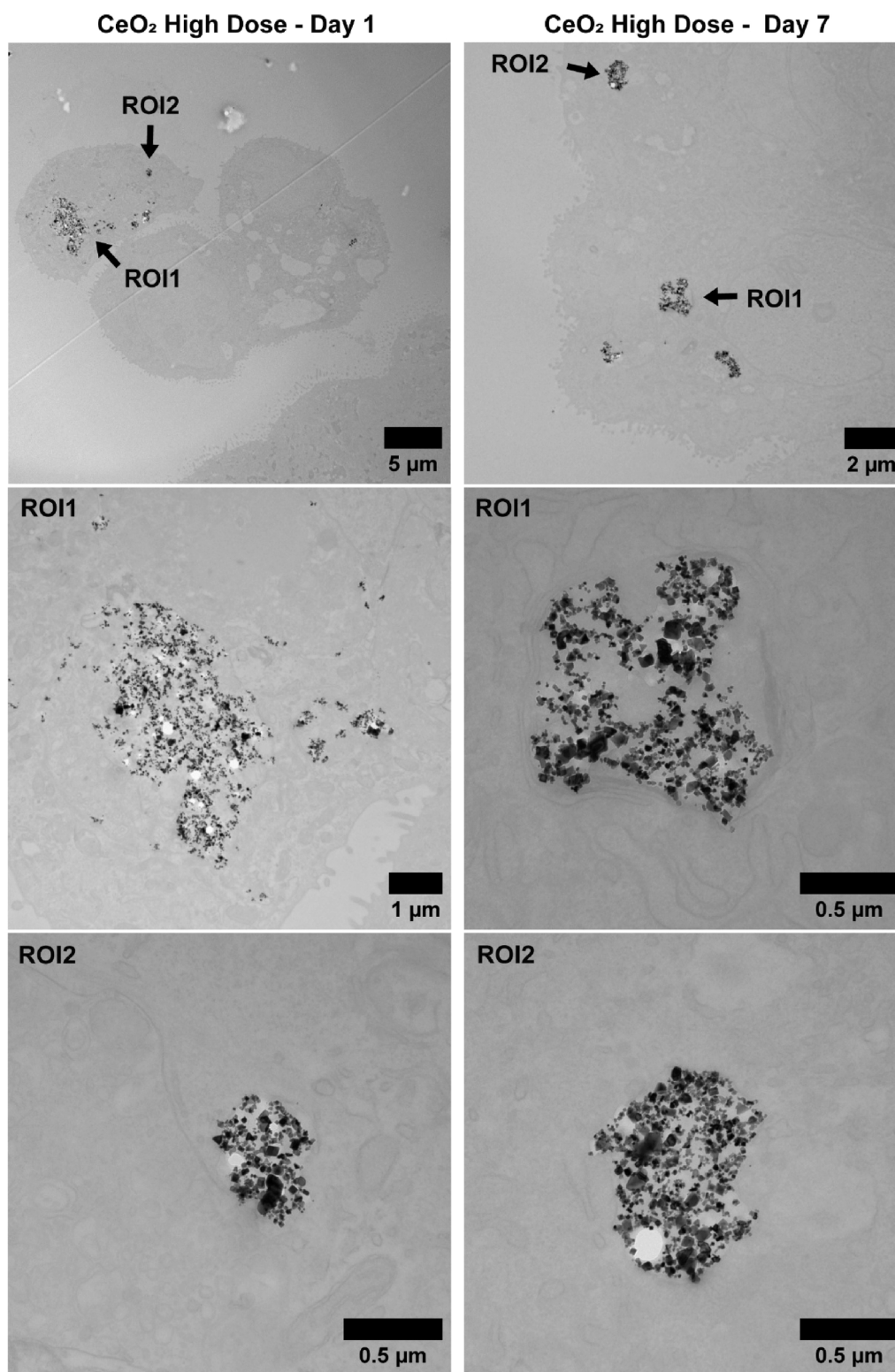


Fig. 2. Intracellular uptake of CeO₂ nanoparticles by MDMs at high dose. Representative TEM micrographs showing CeO₂ particle uptake at day 1 (left) and day 7 (right) at the highest dose (324 μg/cm²). Overview images (top) show particle-laden cells with regions of interest (ROI) indicated by arrows. Magnified ROI panels show NPs accumulated in large membrane-bound vesicles. Scale bars as indicated.

7) to CeO₂ and BaSO₄ in the present study, an approach that again proved essential: the strongest CeO₂-induced cytokine and transcriptional responses emerged only at day 3 and had largely resolved by day 7, a pattern that a conventional acute 24-hour protocol would have missed entirely.

4.2. Dose selection and human relevance

Dose selection for CeO₂ and BaSO₄ was guided by the PATROLS Guidance Document for realistic ENMs lung dosing considerations [13], which derives *in vitro* doses from *in vivo* lung burdens using

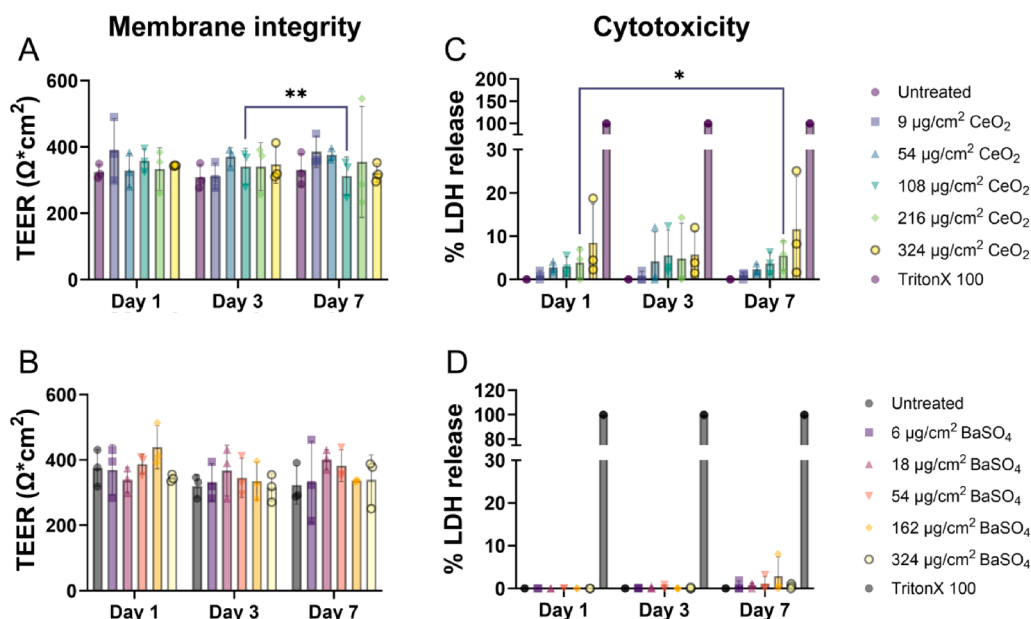


Fig. 3. Assessment of epithelial barrier integrity and cytotoxicity following nanoparticle exposure. (A,B) TEER measurements of co-cultures exposed to increasing concentrations of CeO₂ and BaSO₄ nanoparticles over 7 days. Data are presented as mean $\pm$ standard deviation ($\Omega \cdot \text{cm}^2$), $n = 3$ biological replicates. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test. (C,D) LDH release following CeO₂ and BaSO₄ exposure. Data are presented as mean $\pm$ standard deviation ($n = 3$ biological replicates), normalized to 2% Triton X-100 as 100% and untreated control as 0% LDH release. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$).

material-specific Brunauer-Emmett-Teller (BET) surface areas (27 m^2/g and 33 m^2/g , respectively) and published exposure data. According to this framework, the human-relevant *in vitro* equivalent doses range from 0 to 6 $\mu\text{g}/\text{cm}^2$ for BaSO₄ and from 0 to 9 $\mu\text{g}/\text{cm}^2$ for CeO₂. For context, when the measured lung burdens from a 90-day rat inhalation study [41] are normalized to the proximal alveolar surface area (300 cm^2), the resulting alveolar surface doses at the end of exposure reach approximately 1.6 $\mu\text{g}/\text{cm}^2$ for CeO₂ at 3.0 mg/m^3 and 2.0 $\mu\text{g}/\text{cm}^2$ for BaSO₄ at 50 mg/m^3 , the highest concentrations tested in that study, at which inflammatory responses and impaired clearance were observed. The lowest doses in our study (9 $\mu\text{g}/\text{cm}^2$ for CeO₂; 6 $\mu\text{g}/\text{cm}^2$ for BaSO₄) thus represent the upper bound of this human-relevant range. Doses 30–50 times higher were included as exploratory doses to capture potential threshold or non-linear responses and to simulate upper-bound scenarios such as accidental releases or inadequate respiratory protection.

This positioning is consistent with published *in vitro* work: ALI-exposed co-cultures with CeO₂ NM-212 at doses up to 5 $\mu\text{g}/\text{cm}^2$ [16], and submerged models at up to 9.5 $\mu\text{g}/\text{cm}^2$ [17], reported no significant cytotoxicity or inflammatory cytokine release, and a subchronic ALI study required doses up to 100 $\mu\text{g}/\text{cm}^2$ to detect IL-1 β release [18]. For BaSO₄, doses up to 26.6 $\mu\text{g}/\text{cm}^2$ in ALI-exposed A549 cells produced no significant cytotoxicity [42], while Konduru et al. (2014) demonstrated rapid clearance and low biopersistence *in vivo*, with a pulmonary half-life of approximately 9.6 days [43]. Taken together, these findings confirm that doses at the lower end of our range are unlikely to elicit strong responses in cell line-based systems, further justifying the inclusion of higher exploratory doses to enable robust concentration–response characterization and to simulate upper-bound scenarios such as accidental releases or inadequate respiratory protection.

Having established dose ranges spanning a wide concentration range, we next consider how the physicochemical properties of NPs in biological media may influence their interactions with the cellular model.

4.3. Particle physicochemical properties in biological media and antioxidative capacity

Despite their distinct primary morphologies and physicochemical properties, both materials exhibited broadly similar colloidal behavior in complete cell culture medium (CCM). The hydrodynamic diameters measured by DLS were substantially larger than the primary particle sizes, reflecting the formation of heteroaggregates, which is a well-documented phenomenon in high-ionic-strength, protein-rich media [44]. The formation of a protein corona on both materials was confirmed by the shift of their zeta potential toward strongly negative values in CCM. This charge reversal, particularly striking for CeO₂, reflects extensive adsorption of negatively charged serum proteins, predominantly albumin, onto the particle surface [45,46], which masks the original differences in surface chemistry and gives both materials a similar biological surface profile. This protein corona modulates how each particle interacts with cells and with reactive species in the extracellular environment [44,47]. However, following cellular uptake and trafficking to acidic lysosomal compartments, the protein corona is progressively degraded [45,48,49], re-exposing the native particle surface and allowing intrinsic material properties, such as the redox activity of CeO₂, to drive intracellular biological responses, which are discussed in the following sections.

To assess whether the particles' intrinsic surface reactivity was retained under biological conditions, we performed cell-free DPPH radical scavenging assays in both Milli-Q® water and CCM. CeO₂ displayed concentration-dependent radical scavenging activity consistent with its well-characterized Ce³⁺/Ce⁴⁺ redox cycling [50,51]. In Milli-Q® water, activity increased gradually and plateaued at moderate concentrations. In CCM, meaningful scavenging activity emerged only above approximately 8 mg/mL , corresponding to $\sim 267 \mu\text{g}/\text{cm}^2$ under the exposure conditions used in this study, which is close to the highest tested dose. This suggests that protein corona-mediated masking of redox-active surface sites may limit extracellular reactivity across most of the biologically relevant dose range, while intracellular reactivity following lysosomal protein corona degradation remains the more likely driver of the observed cellular responses. Importantly, most of the doses

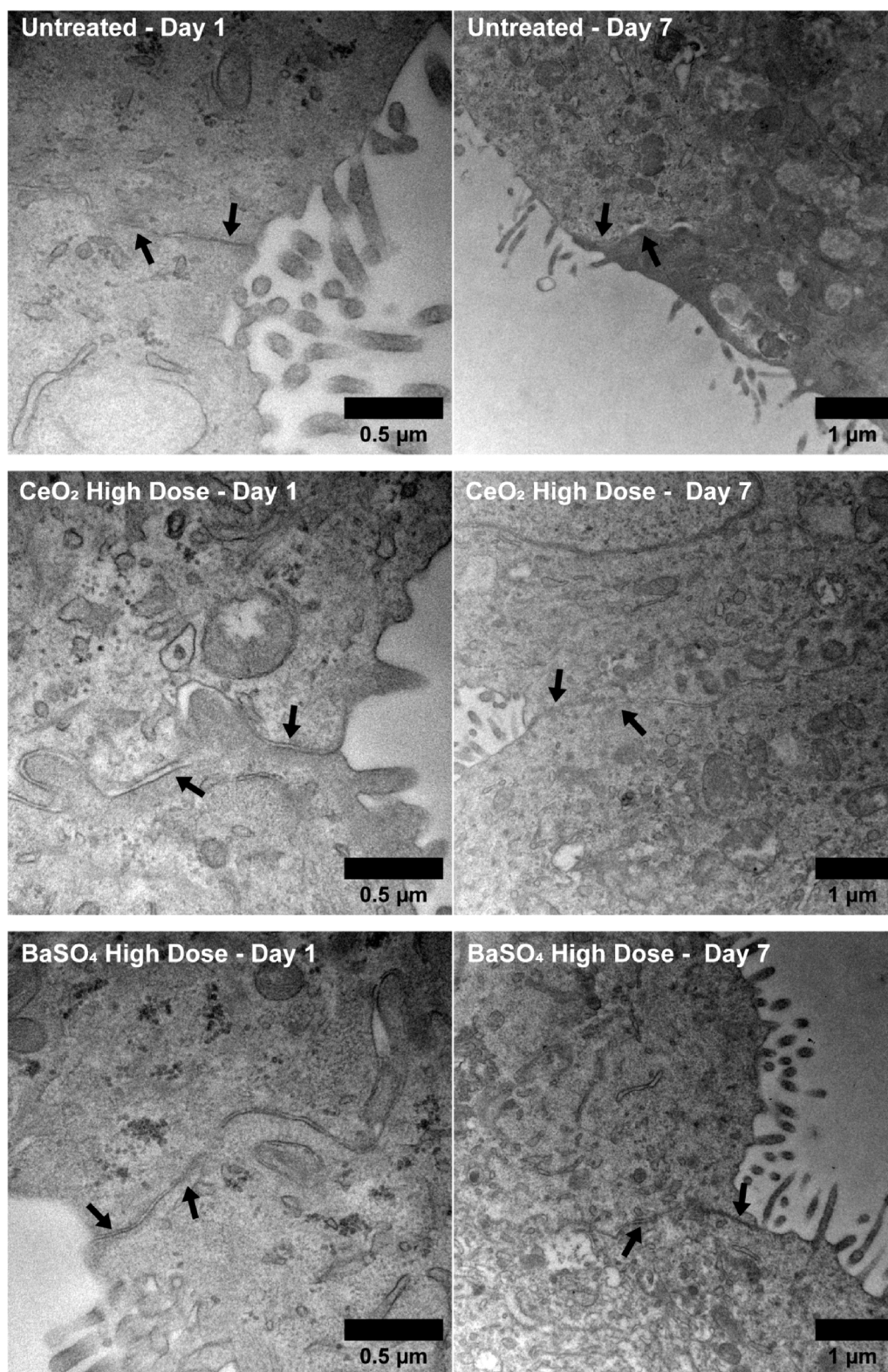


Fig. 4. TEM images of junctional complex in co-cultures exposed to high-dose CeO_2 or BaSO_4 . Representative transmission electron micrographs showing the junctional complex (arrows), comprising tight junctions, adherens junctions, and desmosomes, in untreated co-cultures and co-cultures exposed to the highest dose of CeO_2 ($324 \mu\text{g}/\text{cm}^2$) or BaSO_4 ($324 \mu\text{g}/\text{cm}^2$) at Day 1 and Day 7. Scale bars: $0.5 \mu\text{m}$ (Day 1); $1 \mu\text{m}$ (Day 7).

that produced gene and cytokine responses were below this $\sim 267 \mu\text{g}/\text{cm}^2$ threshold. The biological effects, therefore, occurred at doses that didn't show measurable surface reactivity outside the cell (DPPH assay), pointing to reactivity *inside* the cell (after the protein

corona is degraded in lysosomes) rather than radical chemistry in the surrounding medium.

Notably, while DPPH measurements are widely used to characterize nanoparticle radical scavenging capacity, most published studies report

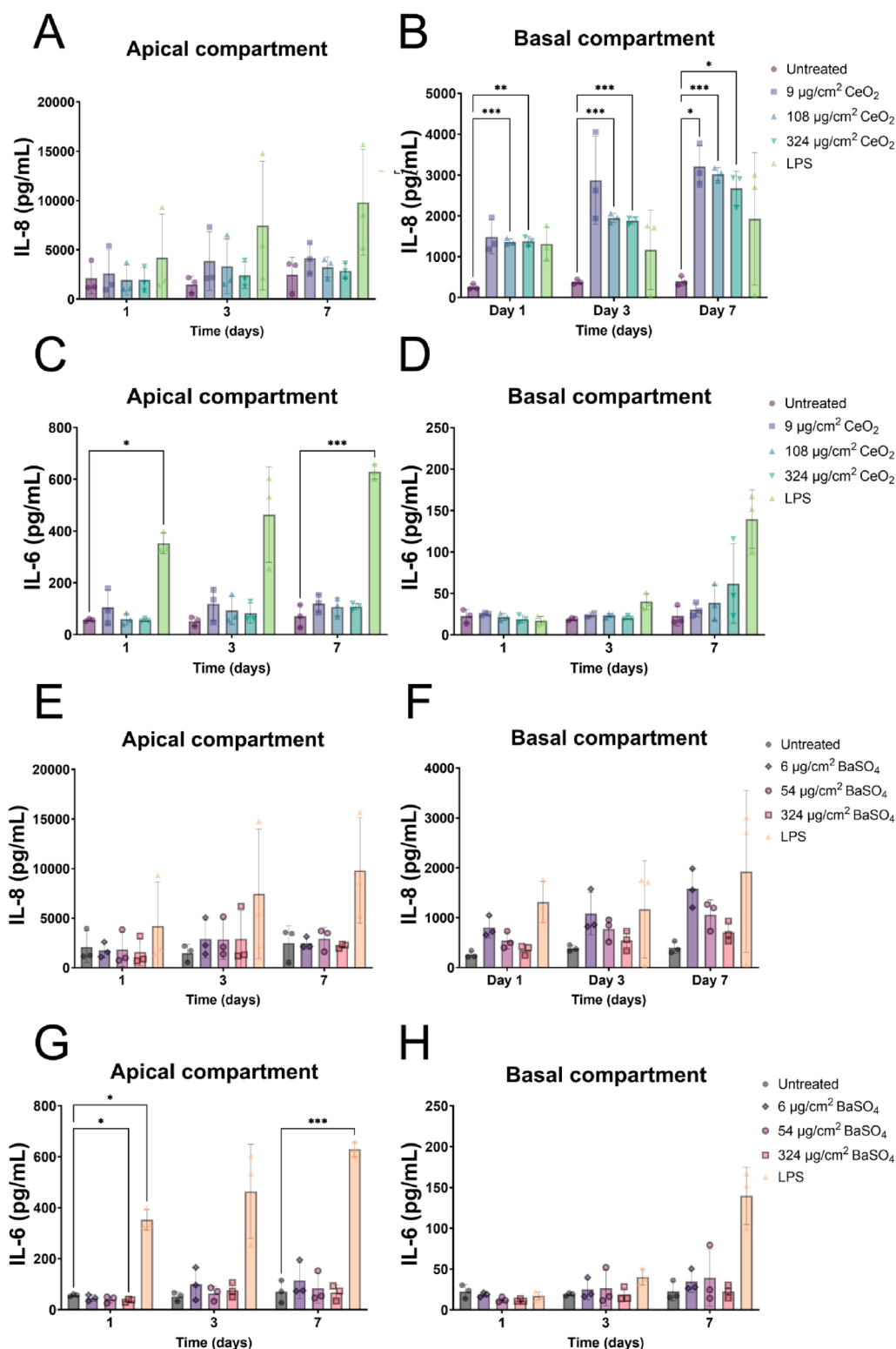


Fig. 5. Inflammatory cytokine secretion in Calu-3/MDMs co-cultures exposed to CeO_2 and BaSO_4 nanoparticles. (A,B) IL-8 and (C,D) IL-6 release from the apical (A, C) and basal (B,D) sides following CeO_2 exposure at different doses and time points. (E,F) IL-8 and (G,H) IL-6 release from the apical (E,G) and basal (F,H) sides following BaSO_4 exposure at different doses and time points. Data are presented as mean $\pm$ standard deviation ($n = 3$ biological replicates). The statistical test performed was a two-way ANOVA with Dunnett's multiple comparisons test, comparing the treated groups to the untreated control of each day. LPS (175 $\mu\text{g}/\text{mL}$) was used as a positive control.

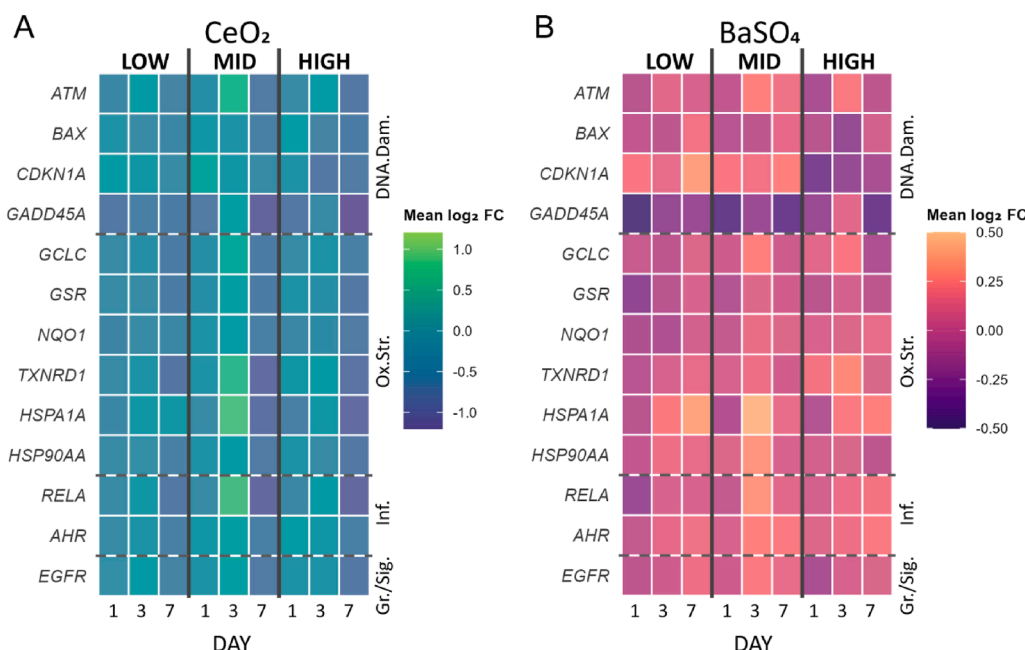


Fig. 6. Transcriptional responses of Calu-3/MDMs co-cultures exposed to CeO₂ (A) and BaSO₄ (B) NPs. Heatmaps showing log₂ fold-change gene expression profiles in Calu-3/MDMs co-cultures following exposure to low (CeO₂: 9 µg/cm²; BaSO₄: 6 µg/cm²), middle (CeO₂: 108 µg/cm²; BaSO₄: 54 µg/cm²), and high (CeO₂/BaSO₄: 324 µg/cm²) doses of NPs over 1, 3, and 7 days. Values are normalized to untreated controls using three housekeeping genes (*GAPDH*, *RPLP0*, *YWHAZ*) and presented as mean (n = 3 biological replicates). Genes include DNA damage/repair markers (DNA. Dam.), oxidative stress markers (Ox. Str.), inflammatory (Inf.), and growth/signaling (Gr./Sig.). Color scales represent log₂ fold change (green/orange = upregulation, blue/purple = downregulation). Comparisons versus the untreated control were performed per gene and timepoint (one-way ANOVA with Dunnett-type comparison, or Kruskal–Wallis with Dunn's test, depending on normality). Values are mean log₂ fold-change versus untreated control (n = 3).

activity in water or organic solvents; measurements in protein-containing cell culture media remain uncommon. Our data demonstrates that surface reactivity assessed under abiotic conditions does not directly predict behavior in biological environments, underscoring the importance of characterizing particle properties under conditions that reflect the actual exposure scenario. The concentration-dependent antioxidant activity of CeO₂ observed here is consistent with results from aqueous-soluble assays such as ABTS (2,2'-azino-bis(3-ethyl-benzthiazoline)-6-sulphonic acid) assay, which have similarly documented CeO₂ radical scavenging in a concentration-dependent manner [52,53].

4.4. Uptake of nanoparticles by cells

TEM was selected to directly confirm particle uptake and sub-cellular localization. The imaging confirmed intracellular particle uptake in both cell types. CeO₂ was predominantly internalized by MDMs, with particle-laden vesicles persisting at both day 1 and day 7, indicating sustained intracellular retention. Notably, particle morphology appeared unchanged between day 1 and day 7, suggesting limited intracellular dissolution and consistent with the known biopersistence of CeO₂ [54]. Calu-3 cells showed markedly lower uptake, consistent with their limited phagocytic capacity. For BaSO₄, only a few intracellular NPs were detected in Calu-3 cells at day 7, which could be explained by the faster intracellular dissolution of BaSO₄ relative to CeO₂ [11]. MDMs were not captured in the BaSO₄ sections, likely due to sampling limitations rather than the absence of uptake.

4.5. Epithelial barrier integrity and cytotoxicity

As a first step in evaluating the biological effects of particle exposure, we assessed standard toxicological endpoints including epithelial barrier integrity (TEER) and cytotoxicity (LDH release). Both NPs preserved epithelial barrier integrity across all tested concentrations and time

points, as evidenced by converging functional (TEER), molecular (*OCN-1* expression), and ultrastructural evidence, with TEM qualitatively confirming the presence of the junctional complex even at the highest doses. Minor but statistically significant decreases in TEER over time were observed at one condition, 108 µg/cm² CeO₂ between day 3 and day 7, however absolute values remained well within the functional range established for this model, and no consistent dose-dependent pattern was observed. This is noteworthy given the wide concentration range tested and the 7-day exposure duration, suggesting that neither particle disrupts the physical barrier even under prolonged exposure. The slight, dose-dependent elevation in LDH release observed with CeO₂, consistent across time points but non-significant, suggests weak membrane perturbation rather than cytotoxicity, a pattern also reported for CeO₂ NM-212 in A549/THP-1 co-cultures at comparable doses [16], with only a minor increase over time observed at 216 µg/cm² between day 1 and day 7. BaSO₄ showed no effect on LDH release at any concentration, consistent with its established low reactivity profile in both *in vitro* [42] and *in vivo* [41] studies.

4.6. Inflammatory responses

To investigate the inflammatory response of the co-culture, we measured IL-8, IL-6, and TNF-α secretion by ELISA in both the apical and basolateral compartments over 7 days of exposure.

Among the cytokines measured, IL-8 showed the clearest and most consistent response to nanoparticle exposure. In polarized airway epithelia, IL-8 is predominantly secreted apically under resting conditions, but basolateral secretion increases during inflammatory activation, facilitating the recruitment of neutrophils from the subepithelial vasculature [55–57]. The significant and sustained increase in basolateral IL-8 secretion observed after CeO₂ exposure at all doses and time points aligns with this established mechanism and highlights IL-8 as a key early event in particle-induced epithelial activation. In contrast, BaSO₄ did not induce IL-8 release to either compartment, supporting its

minimal inflammatory potential.

IL-6 secretion remained near baseline for both materials. In our model, IL-6 concentrations were slightly higher in the apical compartment, consistent with the preferentially apical IL-6 secretion pattern reported for Calu-3 airway epithelia [55,58]. The absence of a dose- or time-dependent increase in IL-6 after CeO₂ exposure may indicate that IL-6-associated inflammatory pathways were not fully engaged at the tested doses, or that IL-6 plays a less prominent role than IL-8 in the early epithelial response to these NPs.

TNF- α was below the detection limit in all conditions. TNF- α is predominantly produced by macrophages, and the relatively low number of MDMs in the co-culture likely limited total secretion below the assay threshold. Additionally, TNF- α mRNA transcription in activated macrophages peaks at approximately 3 h post-stimulation, with protein release following shortly thereafter [57], whereas our earliest sampling occurred at 24 h, by which time TNF- α may already have been cleared or degraded. The absence of detectable TNF- α , therefore, does not contradict the observed IL-8 response; rather, it reflects the model composition and sampling kinetics.

4.7. Transcriptional responses: coordinated activation of oxidative stress, inflammatory, and DNA damage pathways

The transcriptional response to CeO₂ suggests simultaneous, transient activation of oxidative stress defense, inflammatory, proteotoxic stress, and DNA damage pathways, with the strongest effects observed at the middle dose at day 3, though these transcriptional changes did not reach statistical significance and are interpreted as trends. Upregulation of *TXNRD1*, a direct Nrf2 target gene [59] that maintains redox homeostasis by reducing oxidized thioredoxin, is consistent with moderate intracellular reactive oxygen species (ROS) generation, possibly following lysosomal degradation of the protein corona and re-exposure of the redox-active CeO₂ surface. However, the absence of *NQO1* and *HMOX1* upregulation, two other canonical Nrf2 targets, together with unchanged *SOD2*, suggests selective rather than full-spectrum Nrf2 activation, possibly reflecting the moderate nature of the oxidative challenge in this model. *HMOX1* induction has been reported in BEAS-2B cells exposed to CeO₂ under submerged conditions [60], suggesting that the extent of Nrf2 engagement may depend on cell type and exposure conditions.

The concurrent upregulation of *RELA* and *ATM* indicates parallel activation of NF- κ B-mediated inflammatory signaling and p53-associated DNA damage responses. *RELA* upregulation is mechanistically consistent with the sustained IL-8 secretion observed at the protein level, as NF- κ B is a central transcriptional regulator of IL-8 expression [61]. *ATM* activation, together with *GADD45A* induction, reflects engagement of the DNA damage checkpoint machinery, consistent with oxidative stress-driven DNA lesions indicated by *TXNRD1* upregulation. The induction of *HSPA1A* (*HSP70*) further supports a proteotoxic stress response, likely reflecting the cellular burden of managing internalized NPs and the misfolded proteins.

While the broad downregulation of gene expression observed on day 7 could reflect adaptive resolution of the stress response, an alternative explanation is a reduction in the transcriptionally active MDM population under prolonged particle loading. TEM imaging confirmed that MDMs accumulate heavy particle loads as early as day 1, with particle morphology remaining unchanged at day 7, suggesting sustained intracellular burden throughout the exposure period (Fig. 2, Supplementary figure 4). Although MDMs generally maintain viability for up to 7–14 days under standard culture conditions [28], we did not perform cell-type-specific viability assessment at day 7, and therefore cannot distinguish between transcriptional resolution and reduced MDM contribution to the total RNA pool. This represents a limitation of the current dataset and, more broadly, of long-term co-culture particle exposure models.

Given CeO₂'s dual redox behavior, it was particularly important for

us to characterize its intrinsic reactivity before considering its effects in cells, since the literature reports markedly divergent biological outcomes, underscoring the need for careful, context-specific interpretation. Prior work from our group showed that CeO₂ nanoparticles alone modestly suppressed pro-apoptotic gene expression in a human airway model, but when combined with diesel exhaust, amplified the diesel-induced oxidative-stress response, indicating that the same material can shift from a mildly protective to a pro-oxidant role depending on the presence of a co-exposure [62]. A pro-oxidant, cytotoxic profile for CeO₂ alone has also been reported in other human lung epithelial models, including BEAS-2B bronchial epithelial cells [63] and A549 alveolar epithelial cells [64], broadly consistent with the pro-inflammatory, pro-oxidant response we observed here. In contrast, CeO₂ has been reported as protective when applied as a pre-treatment before a separate oxidative challenge, reducing KBrO₃-induced ROS, DNA damage, and cytotoxicity in BEAS-2B cells [65], and as an antioxidant in an intestinal epithelial model, where CeO₂ alone reduced oxidized DNA base levels in differentiated Caco-2 monolayers without affecting barrier integrity [66]. These findings suggest that multiple factors: cell/tissue type, dose, and the presence or absence of a co-occurring oxidative stimulus, contribute to the effect of CeO₂, rather than a fixed pro- or antioxidant character of the material itself, a distinction that is directly relevant to interpreting the sustained, pro-inflammatory response we observed with CeO₂ alone in the present lung model.

In contrast, BaSO₄ elicited minimal transcriptional changes. A non-significant upward trend in *HSPA1A*, *HSP90*, and *RELA* at day 3 (middle dose) may reflect a transient, low-level stress response that did not develop into a coordinated pathway response. One possible explanation is partial intracellular dissolution of BaSO₄ under non-equilibrium lysosomal conditions, releasing barium ions that could contribute to low-level stress signaling [11,67,68]. However, we did not measure BaSO₄ dissolution or barium ion release in this study, so this mechanism is inferred from published data on the same material rather than being directly demonstrated and thus remains speculative. Overall, this is consistent with the generally low reactivity profile of BaSO₄ observed across all other endpoints.

Gene expression was prioritized as the primary readout, in line with the majority of *in vivo* AOP173 benchmarking literature (Table 1) and the broader ALI nanotoxicology field, where transcriptional analysis is favored for its sensitivity, multiplexing capability, and cost-effectiveness [69]. Functional confirmation of these signatures, for example via biochemical assays of oxidative damage or the comet assay for genotoxicity, was beyond the scope of the present study and represents an important direction for future work.

4.8. Alignment of *in vitro* findings with AOP173 key events: CeO₂

The adverse outcome pathway for inflammation-induced pulmonary fibrosis (AOP173) describes a sequence of causally linked key events, from substance interaction with lung resident cell membranes, through inflammation, oxidative stress, and loss of alveolar-capillary integrity, to fibroblast activation and extracellular matrix (ECM) accumulation [27, 70]. We use AOP173 here as an interpretive and comparative framework rather than as an object of validation or development; our aim is to assess the translational relevance of our *in vitro* model by benchmarking its molecular and functional responses against key events already established in the *in vivo* literature. To evaluate the translational relevance of our *in vitro* model, we mapped the markers measured in this study onto the established early key events of AOP173, i.e., oxidative stress, inflammation, and barrier integrity, and compared them with *in vivo* findings from rodent inhalation studies using the same CeO₂ NM-212 material (Table 1).

For the early key events, inflammation, recruitment of inflammatory cells, and oxidative stress, our *in vitro* markers show clear alignment with *in vivo* data, especially for the third day timepoint and middle dose. The sustained basolateral IL-8 secretion and *RELA* upregulation

Table 1Comparison of key event markers in *in vitro* and *in vivo* after CeO₂ exposure.

Key Event / Process	<i>In vivo</i> Markers Reported	<i>In vitro</i> Markers Observed (this study)	Alignment	Representative References for <i>in vivo</i> studies
Inflammation	↑ TNF-α, IL-1β, IL-6, CXCL1/2, CCL2; ↑ neutrophil & macrophage influx; ↑ BALF total protein	↑ IL-8, ↑ RELA; IL-6, TNF-α - unchanged	Partial	[3,41,71,73–75]
Recruitment of inflammatory cells	↑ Neutrophils, macrophages, lymphocytes in BALF; flow cytometry of surface markers	↑ IL-8	Partial	[41,74]
Loss of alveolar-capillary integrity	↑ BALF protein/albumin; histopathology of alveolar damage	TEER largely unchanged; minor isolated decreases at single conditions without a dose-response pattern; OCLN-1 - unchanged. Slight ↑ LDH	Partial	[41,71]
Oxidative Stress	↑ ROS, malondialdehyde (MDA); ↓ SOD, CAT, GSH; ↑ HMOX1, NQO1	↑ HSPA1A (HSP70), ↑ TXNRD1; HMOX1, NQO1 - unchanged	Partial	[3,72,75–77]
Apoptosis / Cell stress	↑ BAX, ↓ BCL-2, ↑ HSP70, DNA damage (COMET assay)	↑ ATM, ↑ HSPA1A (HSP70); BAX - unchanged	Partial	[75,76]
Th2 cell activation / pro-fibrotic signaling	↑ IL-4, ↑ IL-13, ↑ TGF-β1	TGF-β1 - unchanged	None	[41,71]

observed in our model correspond to neutrophil influx, elevated bronchoalveolar lavage fluid (BALF) cytokines (TNF-α, IL-1β, IL-6, CXCL1/2), and macrophage recruitment, as consistently reported in rat inhalation studies at doses as low as 0.3 mg/m³ over 90 days [41,71]. While the specific cytokine profiles differ, reflecting species-dependent chemokine homology (IL-8 is the human functional equivalent of rat CXCL1/CINC-1), the underlying biological process of epithelial-driven neutrophil chemotaxis is conserved. Similarly, the upregulation of *TXNRD1* and *HSPA1A* in our model aligns with elevated ROS, depleted antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and increased heme oxygenase 1 (HMOX1) reported *in vivo* [48,49], confirming oxidative stress as a shared key event across both systems.

Epithelial barrier integrity remained unchanged in our model, with only minor isolated TEER decreases at single conditions and no consistent dose-dependent pattern, contrasting with the histopathological alveolar damage observed *in vivo* at higher doses [41,71]. However, the slight upward trend in LDH levels and the induction of ATM and GADD45A indicate that the model detects early upstream signatures of cellular stress and DNA damage preceding barrier loss in AOP173 progression. The *ATM* and *HSPA1A* responses correspond to the increased *BAX*, decreased *BCL-2*, and elevated *HSP70* reported *in vivo* [72], supporting mechanistic consistency for the apoptosis/cell stress key event. These findings suggest that TEER is an insensitive endpoint for early hazard detection in this model, and that molecular markers of DNA damage and cellular stress, such as *ATM* and *GADD45A*, offer greater sensitivity for capturing AOP-relevant events before barrier disruption.

For the later key events in AOP173, fibroblast proliferation and ECM accumulation, these endpoints were not assessed, as the Calu-3/MDMs co-culture lacks fibroblasts and does not support the chronic exposure durations required for tissue remodeling.

4.9. AOP alignment: BaSO₄ as a low-toxicity benchmark

To further validate the model's specificity, we applied the same AOP173 framework to BaSO₄ (Table 2). Across all key events, BaSO₄ showed minimal or no response, as previously discussed.

Our results closely mirror observations in the *in vivo* literature. In the 90-day rat inhalation study by Schwotzer et al. [3], BaSO₄ at 50 mg/m³, a concentration far exceeding any CeO₂ dose tested, produced minimal inflammation, no persistent effects, and extremely rapid barium clearance with no signs of overload [12]. Other studies confirmed the absence of systemic genotoxicity after 3- and 6-month BaSO₄ inhalation at the same concentration [78], and demonstrated rapid pulmonary clearance, with a half-life of approximately 9.6 days, and predominant translocation of dissolved barium into bone [43]. Importantly, one study on human volunteers showed that acute inhalation of BaSO₄ NPs produced no measurable inflammatory effects, stable lung function, and no evidence of oxidative stress, providing rare human data that supports the low-toxicity classification [79].

The concordance between our *in vitro* findings and these *in vivo* and human data confirms that the Calu-3/MDMs co-culture model correctly identifies BaSO₄ as a low-hazard material. Together with the CeO₂ results, this demonstrates the model's ability to discriminate between NPs

Table 2Comparison of key event markers in *in vitro* and *in vivo* after BaSO₄ exposure.

Key Event / Process	<i>In vivo</i> Markers Reported (rat studies)	<i>In vitro</i> Markers Observed (Calu-3/MDMs, this study)	Alignment	Representative References for <i>in vivo</i> studies
Inflammation	Cytokines unchanged; no BALF increase	IL-8, IL-6 - unchanged	Full	[41,78,79]
Recruitment of inflammatory cells	— (not reported beyond cytokines/BALF inflammation)	IL-8 - unchanged	Full	[41,78]
Loss of alveolar-capillary integrity	— (no BALF protein or injury reported)	TEER largely unchanged; minor isolated decreases at single conditions without a dose-response pattern; OCLN-1 - unchanged LDH - unchanged	Full	[79,80]
Oxidative Stress	No ROS/MDA increase; normal SOD/CAT/GSH	Slight ↑ HSPA1A & HSP90	Partial	[43,80]
Apoptosis / Cell Stress	No BAX/BCL-2 change	Slight ↑ RELA ATM - unchanged	Partial	[43,78]
Th2 cell activation / Pro-fibrotic signaling	— (no IL-4, IL-13, or TGF-β1 changes)	TGF-β1 - unchanged	Full	[41,80]

with fundamentally different hazard profiles based on mechanistic endpoints aligned with AOP173.

5. Conclusion

This study demonstrates that a Calu-3/MDMs co-culture model, exposed under pseudo-ALI conditions across a wide dose range anchored to *in vivo* exposures, can mechanistically distinguish NPs with fundamentally different hazard profiles and capture early key events within AOP173 for inflammation-induced pulmonary fibrosis. CeO₂ produced a coordinated, transient NF- κ B-mediated inflammatory response, most robustly reflected in significant, sustained basolateral IL-8 secretion, accompanied by directional, non-significant transcriptional trends in oxidative stress defense (*TXNRD1*), stress response (*HSPA1A*), and DNA damage signaling (*ATM*, *GADD45A*), peaking at day 3 at the middle dose. Notably, secreted inflammatory signaling was more sensitive than functional barrier measurements: basolateral IL-8 release distinguished CeO₂ from BaSO₄ at doses that produced no measurable change in epithelial barrier integrity (TEER). In contrast, BaSO₄ exhibited minimal activity across all endpoints, consistent with its classification as a low-hazard material and with both *in vivo* rodent data and human volunteer studies. The model proved well-suited for detecting early AOP-relevant responses, while later-stage fibrotic processes fall outside its biological scope and require complementary fibroblast-inclusive systems. These findings support the use of human cell-based models integrated into AOP frameworks as a tool for mechanistic hazard screening of environmentally and occupationally relevant nanomaterials, with the potential to inform regulatory decision-making while reducing reliance on animal testing. The multi-endpoint approach demonstrated here is transferable to other airborne NPs for which mechanistic hazard data from human-relevant systems are lacking, particularly those released into the environment through industrial processes or combustion.

CRediT authorship contribution statement

Jorge Larios: Visualization, Formal analysis. **Sandor Balog:** Writing – review & editing, Investigation, Data curation. **Martina Dzepic:** Writing – review & editing. **Alke Petri-Fink:** Writing – review & editing, Supervision. **Fabian Blank:** Writing – review & editing, Funding acquisition, Conceptualization. **Barbara Rothen-Rutishauser:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Loncarevic Isidora:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Seyran Mutlu:** Writing – review & editing. **Roman Mmanda Fortunatus:** Writing – review & editing, Investigation, Data curation. **Nathalie Jung:** Writing – review & editing, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research is supported by the Swiss National Science Foundation NRP 79 program (Nr. 407940_206331 / 1) and the Adolphe Merkle Foundation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2026.143515](https://doi.org/10.1016/j.jhazmat.2026.143515).

Data Availability

The raw data is available at Zenodo repository on the following link: <https://doi.org/10.5281/zenodo.21298857>

References

- [1] Oberdörster, G., Oberdörster, E., Oberdörster, J., 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect* 113 (7), 823–839.
- [2] Cassee, F.R., van Balen, E.C., Singh, C., Green, D., Muijsers, H., Weinstein, J., et al., 2011. Exposure, health and ecological effects review of engineered nanoscale cerium and cerium oxide associated with its use as a fuel additive. *Crit Rev Toxicol* 41 (3), 213–229.
- [3] Schwotzer, D., Niehof, M., Schaudien, D., Kock, H., Hansen, T., Dasenbrock, C., et al., 2018. Cerium oxide and barium sulfate nanoparticle inhalation affects gene expression in alveolar epithelial cells type II. *J Nanobiotechnol* 16 (1), 16.
- [4] McDonald, J.W., Ghio, A.J., Sheehan, C.E., Bernhardt, P.F., Roggli, V.L., 1995. Rare earth (cerium oxide) pneumoconiosis: analytical scanning electron microscopy and literature review. *Mod Pathol* 8 (8), 859–865.
- [5] Sabbioni, E., Pietra, R., Gaglione, P., Vocaturo, G., Colombo, F., Zanoni, M., et al., 1982. Long-term occupational risk of rare-earth pneumoconiosis A case report as investigated by neutron activation analysis. *Sci Total Environ* 26 (1), 19–32.
- [6] Vocaturo, G., Colombo, F., Zanoni, M., Rodi, F., Sabbioni, E., Pietra, R., 1983. Human exposure to heavy metals. Rare earth pneumoconiosis in occupational workers. *Chest* 83 (5), 780–783.
- [7] TABLE Z-1, Limits for Air Contaminants. Sect. 1910.1000 Table Z-1 (2026).
- [8] Doig, A.T., 1976. Baritosis: a benign pneumoconiosis. *Thorax* 31 (1), 30–39.
- [9] Chong, S., Lee, K.S., Chung, M.J., Han, J., Kwon, O.J., Kim, T.S., 2006. Pneumoconiosis: comparison of imaging and pathologic findings. *Radiographics* 26 (1), 59–77.
- [10] Keller, J.G., Peijnenburg, W., Werle, K., Landsiedel, R., Wohlleben, W., 2020. Understanding Dissolution Rates via Continuous Flow Systems with Physiologically Relevant Metal Ion Saturation in Lysosome. *Nanomaterials* 10 (2), 311.
- [11] Lehner, R., Zanoni, I., Banuscher, A., Costa, A.L., Rothen-Rutishauser, B., 2022. Fate of engineered nanomaterials at the human epithelial lung tissue barrier in vitro after single and repeated exposures. *Front Toxicol* 4, 918633.
- [12] Landsiedel, R., Ma-Hock, L., Hofmann, T., Wiemann, M., Strauss, V., Treumann, S., et al., 2014. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. *Part Fibre Toxicol* 11, 16.
- [13] Tran, L., Barosova, H., Meldrum K., Cliff M.J.D., Rothen-Rutishauser B. Guidance Document for ENMs lung dosing consideration based on in silico analysis for Dörnruper quartz (DQ12), barium sulphate (BaSO₄), cerium oxide (CeO₂), titanium dioxide (TiO₂), and multi-walled carbon nanotubes (MWCNTs). EU: Horizon 2020 PATROLS Project; 2019. Report No.: PATROLS Deliverable 3.1.
- [14] Wiemann, M., Vennemann, A., Sauer, U.G., Wienck, K., Ma-Hock, L., Landsiedel, R., 2016. An in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials. *J Nanobiotechnol* 14, 16.
- [15] Endes, C., Schmid, O., Kinnear, C., Mueller, S., Camarero-Espinosa, S., Vanhecke, D., et al., 2014. An in vitro testing strategy towards mimicking the inhalation of high aspect ratio nanoparticles. *Particle and Fibre. Toxicology* 11 (1), 40.
- [16] Cappellini, F., Di Bucchianico, S., Karri, V., Latvala, S., Malmlof, M., Kippler, M., et al., 2020. Dry Generation of CeO(2) Nanoparticles and Deposition onto a Co-Culture of A549 and THP-1 Cells in Air-Liquid Interface-Dosimetry Considerations and Comparison to Submerged Exposure. *Nanomaterials (Basel)* 10 (4).
- [17] Diabaté, S., Armand, L., Murugadoss, S., Dilger, M., Fritsch-Decker, S., Schlager, C., et al., 2021. Air-Liquid Interface Exposure of Lung Epithelial Cells to Low Doses of Nanoparticles to Assess Pulmonary Adverse Effects. *Nanomaterials* 11 (1), 65.
- [18] García-Salvador, A., Katsumiti, A., Rojas, E., Aristimuño, C., Betanzos, M., Martínez-Moro, M., et al., 2021. A Complete In Vitro Toxicological Assessment of the Biological Effects of Cerium Oxide Nanoparticles: From Acute Toxicity to Multi-Dose Subchronic Cytotoxicity Study. *Nanomaterials (Basel)* 11 (6).
- [19] Di Cristo, L., Maguire, C.M., Mc Quillan, K., Aleardi, M., Volkov, Y., Movia, D., et al., 2018. Towards the Identification of an In Vitro Tool for Assessing the Biological Behavior of Aerosol Supplied Nanomaterials. *Int J Environ Res Public Health* 15 (4).
- [20] Kaur, K., Mohammadpour, R., Sturrock, A., Ghandehari, H., Reilly, C., Paine 3rd, R., et al., 2022. Comparison of biological responses between submerged, pseudo-air-liquid interface, and air-liquid interface exposure of A549 and differentiated THP-1 co-cultures to combustion-derived particles. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 57 (7), 540–551.
- [21] Halappanavar, S., Ede, J.D., Mahapatra, I., Krug, H.F., Kuempel, E.D., Lynch, I., et al., 2021. A methodology for developing key events to advance nanomaterial-relevant adverse outcome pathways to inform risk assessment. *Nanotoxicology* 15 (3), 289–310.
- [22] OECD. Test No. 442C: In Chemico Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins. 2025.
- [23] OECD. Guideline No. 497: Defined Approaches on Skin Sensitisation. Paris; 2025.
- [24] OECD. Test No. 442D: In Vitro Skin Sensitisation: Assays addressing the Adverse Outcome Pathway Key Event on Keratinocyte activation. Paris; 2024.
- [25] OECD. Test No. 442E: In Vitro Skin Sensitisation: In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation. Paris; 2024.

- [26] Halappanavar, S., van den Brule, S., Nymark, P., Gaté, L., Seidel, C., Valentino, S., et al., 2020. Adverse outcome pathways as a tool for the design of testing strategies to support the safety assessment of emerging advanced materials at the nanoscale. *Part Fibre Toxicol* 17 (1), 16.
- [27] Halappanavar S., Sharma M., Solorio-Rodríguez S., Wallin H., Vogel U., Sullivan K., et al. Substance interaction with the pulmonary resident cell membrane components leading to pulmonary fibrosis. 2023.
- [28] He, R.W., Braakhuys, H.M., Vandebriel, R.J., Staal, Y.C.M., Gremmer, E.R., Fokkens, P.H.B., et al., 2021. Optimization of an air-liquid interface in vitro cell co-culture model to estimate the hazard of aerosol exposures. *J Aerosol Sci* 153, 105703.
- [29] Braakhuys, H.M., Gremmer, E.R., Bannusch, A., Drasler, B., Keshavan, S., Rothen-Rutishauser, B., et al., 2023. Transferability and reproducibility of exposed air-liquid interface co-culture lung models. *NanoImpact* 31, 100466.
- [30] Loncarevic, I., Mutlu, S., Dzepic, M., Keshavan, S., Balog, S., Petri-Fink, A., et al., 2026. Alignment of in vitro and in vivo pulmonary inflammation models using crystalline quartz silica. *Part Fibre Toxicol* 23 (1), 6.
- [31] OECD. Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme; First Revision. Paris; 2010. Report No.: ENV/JM/MONO (2009)20/REV.
- [32] PATROLS Consortium. Materials acquisition, physicochemical intrinsic properties and endotoxin evaluation on Tier 1 and 2 ENM. European Commission Horizon 2020, Grant Agreement No. 760813; 2018. Report No.: Deliverable 1.1.
- [33] Keld Alstrup Jensen P.A.C., Renie Birkedal, Yahia Kembouche, Elzbieta Christiansen, Nicklas R. Jacobsen, Marcus Levin, Ismo Koponen, Håkan Wallin, Pieter-Jan de Temmerman and Jan Mast, Camille Guiot and Olivier Spalla, Charles Motzkus, Boris Shivachev, Davy Rousset, Olivier Witschger Standard operating procedures for characterization of the selected manufactured nanomaterials types https://www.anses.fr/en/system/files/nanogenotox_deliverable_5.pdf Accessed 24 Feb 2025 2011 [].
- [34] Basnet, P., Matsuno, T., Neidlein, R., 1997. Potent Free Radical Scavenging Activity of Propolis Isolated from Brazilian Propolis. *Zeitschrift für Naturforschung C* 52 (11-12), 828–833.
- [35] Drasler, B., Karakocak, B.B., Tankus, E.B., Barosova, H., Abe, J., Sousa de Almeida, M., et al., 2020. An Inflamed Human Alveolar Model for Testing the Efficiency of Anti-inflammatory Drugs in vitro. *Front Bioeng Biotechnol* 8, 987.
- [36] Barosova, H., Drasler, B., Petri-Fink, A., Rothen-Rutishauser, B., 2020. Multicellular Human Alveolar Model Composed of Epithelial Cells and Primary Immune Cells for Hazard Assessment. *J Vis Exp* (159).
- [37] Bannusch, A., Schmid, O., Drasler, B., Rohrbasser, A., Braakhuys, H.M., Meldrum, K., et al., 2022. An inter-laboratory effort to harmonize the cell-delivered in vitro dose of aerosolized materials. *NanoImpact* 28, 100439.
- [38] He, R.-W., Braakhuys, H.M., Vandebriel, R.J., Staal, Y.C.M., Gremmer, E.R., Fokkens, P.H.B., et al., 2021. Optimization of an air-liquid interface in vitro cell co-culture model to estimate the hazard of aerosol exposures. *J Aerosol Sci* 153, 105703.
- [39] Kirsty Meldrum S.H.D., and Martin J.D.Clift. Guidance Document for a quasi-air-liquid interface exposure of nanoparticles to cells grown at an air-liquid interface <https://www.patrols-h2020.eu/publications/sops/SOP-library-pdfs/3106-Guidance-Documnt-for-a-quasi-air-liquid-interface-exposure-of-nanoparticles-to-cells-grown-.pdf?m=1636040476&> Accessed 24 Feb 2025 [].
- [40] Sousa de Almeida, M., Taladriz-Blanco, P., Drasler, B., Yajan, P., Petri-Fink, A., et al., 2022. Cellular Uptake of Silica and Gold Nanoparticles Induces Early Activation of Nuclear Receptor NR4A1. *Nanomaterials* (Basel) 12 (4).
- [41] Schwotzer, D., Ernst, H., Schaudien, D., Kock, H., Pohlmann, G., Dasenbrock, C., et al., 2017. Effects from a 90-day inhalation toxicity study with cerium oxide and barium sulfate nanoparticles in rats. *Particle and Fibre Toxicology* 14 (1), 23.
- [42] Küstner, M.J., Eckstein, D., Brauer, D., Mai, P., Hampl, J., Weise, F., et al., 2024. Modular air-liquid interface aerosol exposure system (MALIES) to study toxicity of nanoparticle aerosols in 3D-cultured A549 cells in vitro. *Arch Toxicol* 98 (4), 1061–1080.
- [43] Konduru, N., Keller, J., Ma-Hock, L., Gröters, S., Landsiedel, R., Donaghy, T.C., et al., 2014. Biokinetics and effects of barium sulfate nanoparticles. *Part Fibre Toxicol* 11, 55.
- [44] Moore, T.L., Rodriguez-Lorenzo, L., Hirsch, V., Balog, S., Urban, D., Jud, C., et al., 2015. Nanoparticle colloidal stability in cell culture media and impact on cellular interactions. *Chem Soc Rev* 44 (17), 6287–6305.
- [45] Monopoli, M.P., Aberg, C., Salvati, A., Dawson, K.A., 2012. Biomolecular coronas provide the biological identity of nanosized materials. *Nat Nanotechnol* 7 (12), 779–786.
- [46] Casals, E., Pfaller, T., Duschl, A., Oostingh, G.J., Puentes, V., 2010. Time Evolution of the Nanoparticle Protein Corona. *ACS Nano* 4 (7), 3623–3632.
- [47] Fong, W.-K., Moore, T.L., Balog, S., Vanhecke, D., Rodriguez-Lorenzo, L., Rothen-Rutishauser, B., et al., 2019. Nanoparticle Behaviour in Complex Media: Methods for Characterizing Physicochemical Properties, Evaluating Protein Corona Formation, and Implications for Biological Studies. In: Gehr, P., Zellner, R. (Eds.), *Biological Responses to Nanoscale Particles: Molecular and Cellular Aspects and Methodological Approaches*. Springer International Publishing, Cham, pp. 101–150.
- [48] Bertoli, F., Garry, D., Monopoli, M.P., Salvati, A., Dawson, K.A., 2016. The Intracellular Destiny of the Protein Corona: A Study on its Cellular Internalization and Evolution. *ACS Nano* 10 (11), 10471–10479.
- [49] Wang, F., Yu, L., Monopoli, M.P., Sandin, P., Mahon, E., Salvati, A., et al., 2013. The biomolecular corona is retained during nanoparticle uptake and protects the cells from the damage induced by cationic nanoparticles until degraded in the lysosomes. *Nanomedicine (Lond)* 9 (8), 1159–1168.
- [50] Celardo, I., Pedersen, J.Z., Traversa, E., Ghibelli, L., 2011. Pharmacological potential of cerium oxide nanoparticles. *Nanoscale* 3 (4), 1411–1420.
- [51] Pirmohamed, T., Dowding, J.M., Singh, S., Wasserman, B., Heckert, E., Karakoti, A. S., et al., 2010. Nanoceria exhibit redox state-dependent catalase mimetic activity. *Chemical Communications* 46 (16), 2736–2738.
- [52] Nosrati, H., Heydari, M., Khodaei, M., 2023. Cerium oxide nanoparticles: Synthesis methods and applications in wound healing. *Mater Today Bio* 23, 100823.
- [53] Augustine, R., Zahid, A.A., Hasan, A., Dalvi, Y.B., Jacob, J., 2021. Cerium Oxide Nanoparticle-Loaded Gelatin Methacryloyl Hydrogel Wound-Healing Patch with Free Radical Scavenging Activity. *ACS Biomater Sci Eng* 7 (1), 279–290.
- [54] Keller, J., Wohlleben, W., Ma-Hock, L., Strauss, V., Gröters, S., Kuttler, K., et al., 2014. Time course of lung retention and toxicity of inhaled particles: short-term exposure to nano-Ceria. *Arch Toxicol* 88 (11), 2033–2059.
- [55] Chow, A.W., Liang, J.F., Wong, J.S., Fu, Y., Tang, N.L., Ko, W.H., 2010. Polarized secretion of interleukin (IL)-6 and IL-8 by human airway epithelia 16HBE14o- cells in response to cationic polypeptide challenge. *PLoS One* 5 (8), e12091.
- [56] Carolan, E.J., Mower, D.A., Casale, T.B., 1997. Cytokine-induced Neutrophil Transepithelial Migration Is Dependent upon Epithelial Orientation. *Am J Respir Cell Mol Biol* 17 (6), 727–732.
- [57] Mukaida, N., 2003. Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *Am J Physiol Lung Cell Mol Physiol* 284 (4), L566–L577.
- [58] Sun, Y., Wu, F., Sun, F., Huang, P., 2008. Adenosine Promotes IL-6 Release in Airway Epithelial. *J Immunol* 180 (6), 4173–4181.
- [59] Tonelli, C., Chio, L.L.C., Tuveson, D.A., 2018. Transcriptional Regulation by Nrf2. *Antioxid Redox Signal* 29 (17), 1727–1745.
- [60] Eom, H.-J., Choi, J., 2009. Oxidative stress of CeO₂ nanoparticles via p38-Nrf-2 signaling pathway in human bronchial epithelial cell, Beas-2B. *Toxicol Lett* 187 (2), 77–83.
- [61] Lawrence, T., 2009. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 1 (6), a001651.
- [62] Steiner, S., Mueller, L., Popovicheva, O.B., Raemy, D.O., Czerwinski, J., Comte, P., et al., 2012. Cerium dioxide nanoparticles can interfere with the associated cellular mechanistic response to diesel exhaust exposure. *Toxicol Lett* 214 (2), 218–225.
- [63] Park, E.-J., Choi, J., Park, Y.-K., Park, K., 2008. Oxidative stress induced by cerium oxide nanoparticles in cultured BEAS-2B cells. *Toxicology* 245 (1), 90–100.
- [64] Mittal, S., Pandey, A.K., 2014. Cerium Oxide Nanoparticles Induced Toxicity in Human Lung Cells: Role of ROS Mediated DNA Damage and Apoptosis. *BioMed Res Int* 2014 (1), 891934.
- [65] Rubio, L., Annangi, B., Vila, L., Hernández, A., Marcos, R., 2016. Antioxidant and anti-genotoxic properties of cerium oxide nanoparticles in a pulmonary-like cell system. *Arch Toxicol* 90 (2), 269–278.
- [66] Vila, L., García-Rodríguez, A., Cortés, C., Velázquez, A., Xamena, N., Sampayo-Reyes, A., et al., 2018. Effects of cerium oxide nanoparticles on differentiated/undifferentiated human intestinal Caco-2 cells. *Chem Biol Interact* 283, 38–46.
- [67] Subirana, M.A., Thomas, S., Hause, G., Dobritzsch, D., Glahn, F., Schaumlöffel, D., et al., 2025. Uptake, localization and dissolution of barium sulfate nanoparticles in human lung cells explored by the combination of ICP-MS, TEM and NanoSIMS. *J Trace Elem Med Biol* 89, 127650.
- [68] Keller, J.G., Graham, U.M., Koltermann-Jüly, J., Gelein, R., Ma-Hock, L., Landsiedel, R., et al., 2020. Predicting dissolution and transformation of inhaled nanoparticles in the lung using abiotic flow cells: The case of barium sulfate. *Sci Rep* 10 (1), 458.
- [69] Lenz, A.-G., Karg, E., Brendel, E., Hinze-Heyn, H., Maier, K.L., Eickelberg, O., et al., 2013. Inflammatory and Oxidative Stress Responses of an Alveolar Epithelial Cell Line to Airborne Zinc Oxide Nanoparticles at the Air-Liquid Interface: A Comparison with Conventional, Submerged Cell-Culture Conditions. *BioMed Res Int* 2013 (1), 652632.
- [70] Halappanavar, S., van den Brule, S., Nymark, P., Gaté, L., Seidel, C., Valentino, S., et al., 2020. Adverse outcome pathways as a tool for the design of testing strategies to support the safety assessment of emerging advanced materials at the nanoscale. *Part Fibre Toxicol* 17 (1), 16.
- [71] Guo, C., Robertson, S., Weber, R.J.M., Buckley, A., Warren, J., Hodgson, A., et al., 2019. Pulmonary toxicity of inhaled nano-sized cerium oxide aerosols in Sprague-Dawley rats. *Nanotoxicology* 13 (6), 733–750.
- [72] Srinivas, A., Rao, P.J., Selvam, G., Murthy, P.B., Reddy, P.N., 2011. Acute inhalation toxicity of cerium oxide nanoparticles in rats. *Toxicol Lett* 205 (2), 105–115.
- [73] Nalabotu, S.K., Kolli, M.B., Triest, W.E., Ma, J.Y., Manne, N.D., Katta, A., et al., 2011. Intratracheal instillation of cerium oxide nanoparticles induces hepatic toxicity in male Sprague-Dawley rats. *Int J Nanomedicine* 6, 2327–2335.
- [74] Armstead, A.L., Minarchick, V.C., Porter, D.W., Nurkiewicz, T.R., Li, B., 2015. Acute inflammatory responses of nanoparticles in an intra-tracheal instillation rat model. *PLoS One* 10 (3), e0118778.
- [75] Nemmar, A., Yuvaraju, P., Beegam, S., Fahim, M.A., Ali, B.H., 2017. Cerium Oxide Nanoparticles in Lung Acutely Induce Oxidative Stress, Inflammation, and DNA Damage in Various Organs of Mice. *Oxid Med Cell Longev* 2017, 9639035.
- [76] Dunnick, K.M., Morris, A.M., Badding, M.A., Barger, M., Stefaniak, A.B., Sabolsky, E.M., et al., 2016. Evaluation of the effect of valence state on cerium oxide nanoparticle toxicity following intratracheal instillation in rats. *Nanotoxicology* 10 (7), 992–1000.
- [77] Qian, Q., Zhang, Y., Chen, Y., Ye, C., Feng, Q., Tu, J., et al., 2021. Assessment of pulmonary toxicity of potential antioxidant drug PEGylated nanoceria after intratracheal instillation in rats. *J Appl Toxicol* 41 (6), 941–952.

- [78] Cordelli, E., Keller, J., Eleuteri, P., Villani, P., Ma-Hock, L., Schulz, M., et al., 2017. No genotoxicity in rat blood cells upon 3- or 6-month inhalation exposure to CeO₂ or BaSO₄ nanomaterials. *Mutagenesis* 32 (1), 13–22.
- [79] Monsé, C., Westphal, G., Raulf, M., Jettkant, B., van Kampen, V., Kendzia, B., et al., 2022. No inflammatory effects after acute inhalation of barium sulfate particles in human volunteers. *BMC Pulm Med* 22 (1), 233.
- [80] Molina, R.M., Konduru, N.V., Queiroz, P.M., Figueroa, B., Fu, D., Ma-Hock, L., et al., 2019. Fate of Barium Sulfate Nanoparticles Deposited in the Lungs of Rats. *Sci Rep* 9 (1), 8163.