The curious case of how mimicking physiological complexity in in vitro models of the human respiratory system influences the inflammatory responses. A preliminary study focused on gold nanoparticles

Dania Movia,1* Luisana Di Cristo,1 Roaa Alnemari,2 Joseph E. McCarthy,3 Hanane Moustaoui,4 Marc Lamy de la Chapelle,4 Jolanda Spadavecchia,4 Yuri Volkov1,2,3 & Adriele Prina-Mello1,2,3**

1 Laboratory for Biological Characterization of Advanced Materials (LBCAM), Trinity Translational Medicine Institute, School of Medicine, Trinity College, Dublin, Ireland
2 Department of Clinical Medicine, School of Medicine, Trinity College, Dublin, Ireland
3 CRANN Institute, AMBER Centre, Trinity College, Dublin, Ireland
4 CNRS, UMR 7244, CSPBAT, Laboratoire de Chimie, Structures et Propriétés de Biomateriaux et d’Agents Therapeutiques Université Paris 13, Sorbonne Paris Cité, Bobigny, France CNRS, Paris, France

Keywords
Gold nanoparticles, inflammation, in vitro complexity, monocyte recruitment assay.

Correspondence to: Dania Movia and Adriele Prina-Mello, Full address: Lab 0.74, Trinity Translational Medicine Institute, Trinity Centre for Health Sciences, James’s street, D8, Dublin, Ireland. Tel: 00353 1 896 3259, Fax: 00353 1 896 3037 E-mail: dmovia@tcd.ie; prinamea@tcd.ie

FUNDING INFORMATION
Seventh Framework Programme NANoREG project(grant agreement 310584); Irish Research Council Government of Ireland Postdoctoral Fellowship to DM.

Received: 08 March 2017; Revised: 06 April 2017; Accepted: 20 April 2017

ABSTRACT
Environmental and biomedical nanoparticles can pose potential health risks to the human respiratory system by inducing severe lung inflammation. The aim of this case study is to present a comparison of the inflammatory response in four in vitro models of the human lung epithelium, differing by composition and/or culturing substrates, when exposed to gold nanoparticles (AuNPs). Three in vitro models of lung adenocarcinoma (A549) cells and a commercially available three-dimensional (3D) culture (MucilAir™) were tested. The models were exposed to AuNPs for 3, 6, and 24 h. AuNPs internalisation was investigated by confocal, electron microscopy, and Raman spectroscopy. Enzyme-Linked Immuno-Sorbent Assay (ELISA) was used for quantifying the secretion of the inflammatory mediator Interleukin-6 (IL-6) following exposure to AuNPs. Finally, a microfluidic approach was developed in-house to investigate whether pro-inflammatory mediators present in supernatants harvested from the AuNPs-treated cell cultures could trigger monocyte activation. Our results demonstrated that AuNPs were internalised only in submerged cultures grown on glass substrates. Nevertheless, AuNPs internalisation did not trigger a significant IL-6 secretion. Significant amounts of IL-6 were secreted by AuNPs-treated mono-cultures grown on Transwell™ inserts, triggering monocyte activation in dynamic microfluidic experiments. AuNPs did not induce IL-6 secretion in co-cultures and MucilAir™

© 2017 The Authors. Journal of Interdisciplinary Nanomedicine published by John Wiley & Sons Ltd and the British Society for Nanomedicine This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Introduction

In the last decades, nanotechnology has found application in many medical and industrial fields, raising enthusiasm but also concerns associated with the fact that nanoparticles (NPs) could show toxic effects. Consumers and workers are exposed to NPs from various sources. While biomedical NPs (e.g., drug nanocarriers (Lee, Loo, Traini, & Young, 2015)) are actively introduced into the human body (Bregoli et al., 2016), NPs embedded in commercial goods can be released into the environment accidentally at any stage of the product life cycle, thus coming into contact with human organs and tissues. Hence, nanomaterials risk assessment is considered as an essential prerequisite for any NPs application (Bregoli et al., 2016; Zarogoulidis, Giraleli, & Karamanos, 2012).

Risk assessment studies indeed include investigatory research on the effects of inhaled NPs (Borm & Kreyling, 2004; Geiser & Kreyling, 2010; Jud, Clift, Petri-Fink, & Rothen-Rutishauser, 2013; Klein et al., 2012; Rogueda & Traini, 2007). The human respiratory system is in fact directly connected to the external environment and widely exposed to NPs with applications ranging from lung diseases treatment (Ahmad et al., 2015; Azarmi, Roa, & Lobenberg, 2008; Lu, Zhu, Chen, & Liu, 2014) to pulmonary administration (Mansour, Rhee, & Wu, X., 2009; Muralidharan, Malapit, Mallory, Hayes, & Mansour, 2015; Sung, Pulliam, & Edwards, 2007; Yang et al., 2008), or that are accidentally released into the air during their production or use (Kreyling, Hirn, & Schlehe, 2010; Nazarenko, Han, Lioy, & Mainelis, 2011; Nazarenko, Zhen, Han, Lioy, & Mainelis, 2012; Yokel & Macphail, 2011). A number of local and systemic pathological responses can arise following NPs inhalation (Borm & Kreyling, 2004); these responses are associated with (1) NPs capability to cause inflammation in the lung (local adverse response), and/or (2) NPs translocation into the bloodstream and subsequent systemic toxic effects. Our case study focuses on the adverse effects of NPs remaining in the lung and triggering local inflammation.

Oxidative stress, excessive lung inflammation, and subsequent pulmonary fibrosis are thought to be the key mechanisms driving NPs-induced local adverse responses in the human respiratory system (Duffin, Tran, Brown, Stone, & Donaldson, 2007; Li, Muralikrishnan, et al., 2010; Mohamud et al., 2014; Muhlfeld et al., 2008; Schinwald et al., 2012; Walling & Lau, 2014). Such inflammatory mechanisms have been thoroughly investigated and elucidated. To date, it is commonly accepted that the NPs physico-chemical properties (such as composition, size, surface charge, and agglomeration) (Braakhuis, Park, Gosens, De Jong, & Cassee, 2014; Kim et al., 2016; Kreyling, Semmler-Behnke, Takenaka, & Moller, 2013; Madl, Plummer, Carosino, & Pinkerton, 2014; Rotoli et al., 2015), as well as the timing (acute vs. chronic) and route (e.g., intratracheal or intranasal) of administration (Landsiedel, Sauer, Ma-Hock, Schneekenburger, & Wiemann, 2014; Morimoto et al., 2016), strongly influence the lung inflammatory response to NPs (Mohamud et al., 2014). Most of these paradigms have been extrapolated from the data originating from animal studies, which are widely used for evaluating inflammatory responses in inhalation toxicology studies. The use of animal models is however constantly raising ethical concerns and research costs. In addition, animal models do not comprehensively mimic the human body, and this entirely holds true in relation to the histology of the human respiratory system (Gordon et al., 2015; Hayes & Bakand, 2014; Pauluhn & Mohr, 2000). Rodents are the main animal model used in inhalation toxicology studies (Pauluhn, 2003); yet, the pulmonary anatomy of these animals significantly differs from that of humans (Phalen, Oldham, & Wolff, 2008). Major differences can be observed in the upper airways: humans have a simple nasal anatomy, whereas rats have a highly complex set of ethmoid turbinates (Frohlich & Salar-Bezhadi, 2014). Mice, hamsters, rabbits, and dogs share a similar morphology. In addition, there are significant differences in the distribution of epithelial cell populations along the respiratory tract and in the histology of the connective
tissue. Finally, although intratracheal instillation is widely accepted as a useful method of animal exposure in nanosafety research (Kreyling et al., 2010; Morimoto et al., 2016), it produces a NPs lung deposition that is heterogeneous and far from physiological (Oberdorster, 2010). Lung inflammation is indeed affected by the architecture of the respiratory system and by NPs deposition pattern. Thus, uncertainties are looming over the inflammation paradigms extrapolated from animal inhalation studies (Landsiedel et al., 2014). Because of these reasons, replacing animal models with more tissue-mimetic in vitro models of the human respiratory system is attracting the interest of the nanosafety and nanomedicine research communities. Various studies and scientific reports have aimed at offering innovative solutions in this context (Leong & Ng, 2014; Rothen-Rutishauser, Blank, Muhlfeld and Gehr, 2008; Zhang & Khademhosseini, 2015). However, the authors believe that knowledge gaps still exist on how to select the most physiologically representative in vitro testing models capable to appropriately imitate inflammatory responses caused by inhaled NPs. Our case study aims at providing an interesting insight into the in vitro parameters that should be taken into account during such selection process.

AuNPs were selected as a subject of this case study, because they are a candidate of extreme interest for the nanomedicine community (Ashraf et al., 2016; Bregoli et al., 2016). The application of gold nanomaterials as drug carriers for the treatment of lung adenocarcinoma (Movia et al., 2014) and pancreatic cancer (Spadavecchia et al., 2016) has been recently reported by some of the authors. Although it has been demonstrated that NPs formed by inorganic metals generally induce inflammation in the lung (Mohamud et al., 2014), AuNPs seem to constitute an exception from this rule. Inhaled AuNPs are in fact often associated with inflammation inhibition (Jacobsen et al., 2009), as well as with poor translocation from the lung to distant organs (Sung et al., 2011), making them perfect candidates as drug carrier for lung diseases treatment. A significant AuNPs-induced down-regulation of the inflammatory signals has also been detected in other organs and cell models (Chen et al., 2013; Khan, Abdelhalim, Alhomida, & Al-Ayed, M. S., 2013; Selim, Abd-Elhakim, & Al-Ayadhi, 2015; Sumbayev et al., 2013; Uchiyama et al., 2014; Villiers, Freitas, Couderc, Villiers, & Marche, 2010). This could potentially open novel therapeutic options for the use of AuNPs as anti-inflammatory agents. Notably, all these effects have been found to be strongly influenced by preexisting health and inflammatory conditions in the lungs (Hussain et al., 2011; Jacobsen et al., 2009); whereas, agglomeration of inhaled AuNPs (Balasubramanian et al., 2013; Gosens et al., 2010) or their interaction with lung surfactant proteins (e.g., surfactant protein D) (Schlehe et al., 2013) do not play any role in defining the anti- or pro-inflammatory action of AuNPs in the lungs. Although in general, the scientific literature seems to demonstrate that AuNPs inhibit inflammation, controversial reports can still be found. For example, when monitoring the influence of AuNPs on in vitro phagocyte functions in the lung, both an increased and an unaffected cytokine secretion have been detected (Frohlich, 2015). Although according to Chen et al. no pulmonary inflammation was detectable after short exposure to AuNPs (Chen, Hung, Liu, & Huang, 2009), it has been reported that long-term (90 days) exposure promoted alveolar inflammation in rats (Sung et al., 2011). Other studies indicated that 10-50 nm AuNPs triggered lung emphysema and subsequent animals’ death when intraperitoneally injected into mice (Chen et al., 2009). Notably, AuNPs-induced lung inflammation has been found to be dependent on surface chemistry. For example, a pro-inflammatory response has been reported for epithelial airway models exposed to AuNPs coated with bovine serum albumin (Rothen-Rutishauser, Muhlfeld, Blank, Musso, & Gehr, 2007). Although this and other important AuNPs physico-chemical parameters play a key role in defining the inflammatory effect of such nanomaterials (Mohamud et al., 2014; Seydoux et al., 2016; Tian et al., 2015), the authors believe that one of the main reasons behind the controversial results presented in the scientific literature might originate in the experimental models used.

With this working hypothesis in mind, the in vitro cultures used in our case study differed in complexity (e.g., mono-culture vs. co-culture of multiple cell types; immortalized cells vs. reconstituted tissue; submerged vs. Air-Liquid Interface culturing conditions) or culturing substrate (glass vs. polyethylene terephthalate (PET) Transwell™ inserts). Our aim was to understand how such differences could affect the detectable inflammation triggered by AuNPs. Inflammatory responses were quantified by Interleukin-6 (IL-6) secretion in Enzyme-Linked Immuno-Sorbent Assay (ELISA) and by monocyte activation in a microfluidic set-up developed in our centre.
Interestingly, such responses were not associated with AuNPs cellular internalisation, while they were deeply influenced by the culturing substrate used. AuNPs pro-inflammatory action was also inversely correlated to the complexity of the in vitro model tested.

Materials and Methods

Cell culture

Human adenocarcinoma cells (A549 cell line), human lung fibroblasts (MRC-5 cell line), and human monocyctic leukaemia cells (THP-1 cell line) were obtained from the American Tissue Culture Collection (LG Standards, Teddington, Middlesex, UK). A549 and THP-1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM), while MRC-5 cells were cultured in Modified Eagle Medium (MEM). Cell culture media (Gibco, Invitrogen, Bio-Sciences Ltd, Ireland) were supplemented with 1% penicillin/streptomycin (Gibco, Invitrogen, Bio-Sciences Ltd, Dublin, Ireland) and 10% Fetal Bovine Serum (Sigma-Aldrich, Dublin, Ireland). Cells were incubated at 37°C and 5% CO2. The passage number of the A549 cells was restricted between 7 and 20, whereas for MRC-5 cells and THP-1 cells the used passage number ranged between 5 and 15, and 8-10, respectively. For cell seeding, cells were detached from cell-culture flask substrate with Tryple™ (Gibco, Invitrogen, Bio-Sciences Ltd, Dublin, Ireland), centrifuged, counted using a Countess™ Automated Cell Counter (Invitrogen, Bio-Sciences Ltd, Dublin, Ireland) and diluted in the supplemented media at concentration appropriate for each experiment. The seeding concentration of A549 cells was kept constant among all cell models (1.2 x 10^4 cells/cm^2). MRC-5 cells were diluted in the supplemented media at concentration of 4 x 10^5 cells/mL. For the monocyte activation assay, THP-1 cells were used at concentration 1.6 x 10^6 cells/mL.

Submerged cell culture model

A549 cells were seeded on unmodified glass coverslips (diameter: 13 mm) placed in 24-well plates (final volume/well: 100 µL/well of cell suspension and 600 µL/ well of fresh media), following a protocol previously reported (McIntyre et al., 2016; Verma et al., 2012). Cells were incubated for 2 h at 37°C (5% CO2) to allow cell attachment to the glass substrate.

The curious case of in vitro complexity

Mono-cultures on Transwell™ membranes

A549 cells were seeded on Transwell™ Permeable Supports with PET membrane inserts of 6.5 mm of diameter (growth area: 0.33 cm^2) and pore size of 0.4 µm (Corning Costar, VWR International, Dublin, Ireland) accordingly to supplier’s protocol. Briefly, fresh medium was first added to the wells of a 24-well plate (600 µL), then the Transwell™ inserts were inserted into the wells and plates incubated for 2/3 h at 37°C to favour cell attachment and growth. Cells were then added to the apical compartment of the Transwell™ inserts (final volume: 100 µL/insert), and incubated at 37°C and 5% CO2 for 24 h to allow cell attachment.

Co-culture models on Transwell™ membranes

A co-culture model was formed by co-culturing A549 cells in the presence of an excess of fibroblasts (MRC-5 cells) on the apical side of Transwell™ Permeable Supports with PET membrane inserts of 6.5 mm of diameter (growth area: 0.33 cm^2) and pore size of 0.4 µm (Corning Costar, VWR International, Dublin Ireland). Firstly, fresh supplemented MEM medium was added to the well (600 µL) of a 24-well plate, the Transwell™ inserts added, and plates incubated for 2/3 h at 37°C to favour cell attachment and growth. A total of 100 µL/insert of the MRC-5 cell suspension was then added to the apical compartment (final seeding concentration: 1.2 x 10^5 cells/cm^2); cells were incubated for 48 h to allow cell attachment and spreading. After 48 h, the media in the basolateral compartment was replaced with fresh MEM medium (600 µL/well), and A549 cells were seeded on the top of the MRC-5 cells in DMEM media (final volume: 100 µL/insert). The seeding A549 : MRC-5 ratio was 1 : 10. Prior to seeding, A549 cells were stained with 20 mM Cell Tracker™ Green CMFDA (Invitrogen, Bio-Sciences Ltd, Dublin, Ireland) for 45 min at 37°C and 5% CO2 to allow their identification in co-culture models. Plates were incubated for 24 h to allow the successful formation of the co-culture models.

MucilAir™ cultures

A commercial three-dimensional (3D) human airway epithelial culture model (called MucilAir™) was kindly supplied by Epithelix Sarl (Geneva, Switzerland). MucilAir™ models are characterised by a pseudostratified columnar epithelium presenting beating cilia and mucus production. A representative video of the beating cilia is reported in the Supporting Information (Video S1). The MucilAir™ model mimics
the upper respiratory tract structure of the human lung, including basal, goblet, and ciliated cells. MuclAir™ cultures used in his study were originated from primary human cells isolated from the human nasal cavity of a human donor. According to the supplier’s certificate of analysis, the donor for the cultures was a 40-year old Caucasian woman, with no pathology reported (batch number: MD047101). MuclAir™ cultures were supplied on Corning Costar Transwell™ Permeable Supports with PET membrane inserts of 6.5 mm of diameter and pore size of 0.4 μm. MuclAir™ models were cultured at the Air-Liquid Interface (ALI) in a 24-well plate at 37°C and 5% CO₂ with a specific culture medium (MuclAir™ Culture Medium, Epithelix Sarl, Geneva, Switzerland). The MuclAir™ apical side was washed prior to AuNPs exposure, according to the supplier’s recommendations, to remove the mucus produced by goblet cells overtime.

In vitro exposure to gold nanoparticles and cellular internalisation

Synthesis and properties of gold nanoparticles
Gold nanoparticles (ØTEM = 12.5 ± 1.0 nm; hydrodynamic diameterDLS in deionized water = 14.5 ± 5 nm) were synthesised in sterile water according to a previously published protocol (Sivaraman, Kumar, & Santhanam, 2011). Briefly, 0.25mL of 0.254 mM of HAuCl₄ (Sigma-Aldrich, Dublin, Ireland) was added to a 24.75 mL boiling solution of trisodium citrate (Sigma-Aldrich, Dublin, Ireland) in sterile water at a trisodium citrate to HAuCl₄ molar ratio of 5 : 1. The synthesised particles were left to cool, washed over a nanoporous filter, and re-suspended in sterile water. AuNPs characterisation by Transmission Electron Microscopy (TEM) is reported in Figure S2 in the Supporting Information. AuNPs were tested for endotoxin presence: the chromogenic Pierce® LAL Chromogenic Endotoxin Quantitation kit (Thermo Scientific, Dublin, Ireland) did not detect any significant amount of endotoxin in the AuNPs sample used in this study (Fig. S3 in the Supporting Information). The AuNPs aqueous suspension was diluted in supplemented cell culture medium for further testing.

Exposure to gold nanoparticles
The AuNPs doses tested were kept comparable among the in vitro models under investigation. In order to achieve this, the different growth area between the two culture substrates used (coverslips = 1.76 cm²; Transwell™ membranes = 0.33 cm²) was kept into account. Cell cultures were exposed to AuNPs at a nontoxic particle dose equal to 0.06 μg/cm². Submerged cultures were exposed to a AuNPs dispersion at Au metal concentration of 13 pM (corresponding to 0.2 μg/mL). A total of 500 μL were added to each well. Cell mono-cultures and co-cultures grown on Transwell™ membranes were exposed to AuNPs by adding 100 μL/insert of AuNPs dispersion (13 pM) to the apical compartment. MuclAir™ cultures were exposed to 0.06 μg/cm² by adding 30 μL of AuNPs dispersion (44 pM) in saline (0.9%NaCl + 1.25 mM CaCl₂ + 10 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid)) to the apical compartment of the Transwell™ inserts. Cell models were exposed to AuNPs for 3, 6 and 24 h.

Immunocytochemistry and Laser Scanning Confocal Microscopy
After exposure to AuNPs, cell cultures were fixed with 3.7% paraformaldehyde (Sigma-Aldrich, Ireland) for 10 min at ambient temperature and permeabilized with 0.1% Triton-X (Sigma-Aldrich, Dublin, Ireland) for 3 min. MuclAir™ cultures were fixed with 3.7% paraformaldehyde for 30 min at ambient temperature and permeabilized with 0.5% Triton X for 2 h. Bovine serum albumin (Sigma-Aldrich, Dublin, Ireland) at 1% in Phosphate-Buffered Saline (PBS) (Fisher Scientific Ireland, Dublin, Ireland) was used to block unspecific staining. A 30 min incubation time was used in all the cell models, excluding MuclAir™ cultures that were blocked for 2 h. Cells were stained with Hoechst 33342 (Invitrogen, Bio-Sciences Ltd, Dublin, Ireland) for nuclei, rhodamine phalloidin (Invitrogen, Bio-Sciences Ltd, Dublin, Ireland) for F-actin filaments and mouse anti-α tubulin Alexa 488 (Invitrogen, OR) for tubulin microtubules; co-cultures were exclusively stained with Hoechst 33342 and rhodamine phalloidin. Cell culture models were incubated with the staining solution for 2 h at ambient temperature. After multiple washes with PBS, coverslips and Transwell™ inserts were mounted on glass slides with transparent mounting medium (VECTASHIELD, Vector Laboratories Inc., CAD, USA) and sealed. AuNPs cellular internalisation was evaluated by Laser Scanning Confocal Microscopy (LSCM). LSCM imaging and analysis was carried out with a ZEISS 510 Meta confocal microscope equipped with a Zeiss LSM 5 software (Carl Zeiss Microscopy GmbH, Jena, Germany). AuNPs were imaged in reflectance mode.
at $\lambda_{\text{exc}} = 561$ nm, as previously reported (Movia et al., 2014). AuNPs are well known to scatter light strongly (Aslan, Lakowicz, & Geddes, 2005; Jain, Lee, El-Sayed, & EL-SAYED, 2006; Lee & El-Sayed, 2005; Lee & El-Sayed, 2006; Orendorff, Sau, & Murphy, 2006; Yguerabide & Yguerabide, 1998), making their detection by LSCM possible.

Raman spectroscopy and dark-field microscopy of submerged cultures

Untreated and AuNPs-exposed (24 h) submerged cultures of A549 cells on glass substrates were examined by Raman spectroscopy. Cell cultures were fixed at ambient temperature in 2.5% glutaraldehyde (Sigma-Aldrich, Dublin, Ireland) in PBS for 10 min and then washed multiple times with PBS. Raman spectroscopy measurements were carried out by means of a Xplora spectrometer (Horiba Scientifics, NJ, USA) at excitation wavelength of 660 nm at ambient temperature. A 100× objective was used with a numerical aperture of 0.9 in backscattering configuration. The achieved spectral resolution was close to 2/cm. As a control, the Raman spectrum of AuNPs in aqueous solution was also acquired.

Transmission Electron Microscopy of biological specimens

Transmission Electron Microscopy images of ultrathin sections of the tested cell models were examined. After exposure to AuNPs for 24 h, cell cultures were fixed at ambient temperature in 2.5% glutaraldehyde, and specimens were prepared as previously described (Movia et al., 2014). After mounting on 300-mesh Cu grids, these sections were stained with uranyl acetate and lead citrate and finally imaged (Tecnai Transmission Electron Microscope, FEI, Oregon, USA).

Cytokines secretion

The production of the pro-inflammatory cytokine IL-6 was evaluated and quantified by Sandwich ELISA (Human IL-6 ELISA MAX™ Standard Sets, BioLegend, MSC, Dublin, Ireland). Some of the authors have previously demonstrated that A549 cells can secrete IL-6 in response to exposure to nanomaterials with inflammatory potential (Mohamed et al., 2013). Similarly, IL-6 has demonstrated to be a predictive marker of inflammatory response to respiratory sensitizers in in vitro reconstituted 3D human lung epithelial (MucilAir™) models (Huang, Wiszniewski, Constant, & Roggen, 2013). Supernatants harvested from in vitro models exposed to AuNPs at a particle dose equal to 0.06 μg/cm² for 3, 6, and 24 h were tested. Supernatants of negative controls (untreated cell models; NT) were also tested for comparison. For cell cultures grown on Transwell™ Permeable Supports, the concentration of IL-6 is reported only for the supernatants isolated from the apical compartment, as no detectable levels of IL-6 could be found in the supernatants isolated from the basolateral compartment (Fig. S4). This result suggests that there was a polarised IL-6 secretion by the epithelial cells into the apical compartment. This has been previously reported by other research groups for various human lung cell lines (including A549 cells) grown on Transwell™ membranes (Carolan, Mower, & Casale, 1997; Chow et al., 2010; Sun, Wu, Sun, & Huang, 2008). In order to include a positive control (PT) in the experimental design, the in vitro models were exposed to lipopolysaccharides (LPS) (Sigma-Aldrich, Dublin, Ireland) at concentration of 200 ng/mL for 6 h and the respective supernatants then tested for IL-6 content. LPS is known to trigger inflammatory responses (Abate, Alghaithy, Parton, Jones, & Jackson, 2010) and IL-6 secretion (Relja et al., 2014; Xie et al., 2009) in A549 cells. ELISAs were carried out according to the manufacturer’s manual and samples tested in duplicate. The Epoch microplate reader (Biotek, Mason Technology Ltd, Dublin, Ireland) was used to detect the optical density at 450 nm for each well, and the determined values were corrected by subtracting the optical aberration of the 96-well plastic plate at 570 nm; the means of the resulted values were calculated and calibrated against a standard curve. Separating the used AuNPs from the extracted supernatants is a complicated procedure. In order to account for potential optical interference of AuNPs with the ELISA read-outs, the IL-6 standard was dissolved in the assay diluent (as for manufacturer’s protocol) or in assay diluent spiked with an AuNPs in supplemented DMEM media at concentration of 13 pM. This allowed evaluating whether AuNPs present in the tested supernatants might interfere with the ELISA. It was found that the particles affected the IL-6 ELISA readout, quenching the absorbance signal, as showed by the difference in the resulting calibration curves reported in Figure S5 in the Supporting Information. Hence, cytokines concentrations in supernatants were extrapolated considering the two ELISA calibration curves and the presence or not of AuNPs in the samples tested.
The curious case of in vitro complexity

**Monocyte adhesion assay in dynamic microfluidic environment acting as inflammation recruitment model**

The response of human monocytes (THP-1 cells) to pro-inflammatory signals secreted by AuNPs-treated in vitro models was analysed qualitatively and quantitatively in a microfluidic environment. *Vena8* biochips (Cellix Ltd, Dublin, Ireland) were used (Konya, Mcgettrick, & Heinemann, 2014; Munir et al., 2015; Robinson et al., 2009). The biochip includes eight micro-channels, which are 28.00, 0.80, and 0.12 mm in length, width, and height, respectively. The micro-channels were coated with recombinant human Vascular Cell Adhesion Molecule-1 (rhVCAM-1) (Sigma-Aldrich, Dublin, Ireland) at concentration of 0.03 μg/cm² for 24 h. As a PT for the assay, THP-1 cells were mixed at a ratio of 1 : 1 with recombinant human Monocyte Chemoattractant Protein-1 (rhMCP-1) (Sigma-Aldrich, Dublin, Ireland) at concentration of 200 μg/mL in PBS (final volume: 10 μL/channel) one day prior the assay and incubated at 4°C overnight. THP-1 cells at concentration of 1.6 × 10⁶ cells/mL were mixed with the supernatants harvested from untreated or AuNPs-treated in vitro models at ratio of 1 : 1 and incubated at 37°C and 5% CO₂ for 30 min to allow THP-1 cell activation processes. In the case of cultures grown on Transwell™ inserts, the supernatants collected from the apical compartments were used. The final pool of supernatants tested were harvested from: untreated mono-cultures and co-cultures grown on Transwell™ inserts and MucilAir™ models, and the respective AuNPs-treated models exposed at concentration of 0.06 μg/cm² for 24 h. As a PT for the assay, THP-1 cells were mixed at a ratio of 1 : 1 with recombinant human Monocyte Chemoattractant Protein-1 (rhMCP-1) (Sigma-Aldrich, Dublin, Ireland) at concentration of 1 μg/mL and incubated at 37°C and 5% CO₂ for 30 min. Untreated THP-1 cells were included in the experimental design as negative control (NT). Further controls were used to test the robustness of the chosen experimental set-up, such as the use of uncoated channels. The experiments under dynamic conditions were performed based on slightly adapted protocols publicly available from Cellix Ltd and that have been used in a number of studies (Choi et al., 2008; Dominical et al., 2015; Ferkau et al., 2013; Long et al., 2004; Munir et al., 2015; Nissinen et al., 2010; Robinson, Kashanin, O’Dowd, Williams, & Walsh, 2008; Wu, Mitchell, & Walsh, 2005). Briefly, after washing the channel with 30 μL DMEM media, THP-1 cells were introduced at shear stress of 0.03 dyne/cm². A neMESYS™ syringe pump (Cetoni GmbH, Korbussen, Germany) controlled by the neMESYS UserInterface software was used. Cells flow was monitored with a NIKON TE 300 Eclipse epifluorescence microscope (20× objective lens). Brightfield images were continuously acquired at rate of 1 frame every 10 ms for a minute (for a total of 360 frames) by QImaging software (Media cybernetics, Cambridge, UK). Representative videos of the experiments carried out in the dynamic microfluidic environment are reported as Supporting Information (Video S2–S10). When visioning the supporting material, attention should be focused on the cells adhering to the channels: these cells are activated monocyte, and their number correlates with the inflammmogenic stimulus. Every sample was run in duplicate on two different rhVCAM-1 coated channels and imaged at three adjacent locations. Every sample was perfused for a total time of 6 min. For quantitative analysis, every video recorded was analysed, and adherent cells present in every chosen location were counted manually.

**Statistical analysis**

Graph-Pad Prism (Graph-Pad Software Inc., La Jolla, CA, USA) was used to carry out the statistical analysis. A *P* value < 0.05 was considered statistically significant. The statistical tests used are specified in the corresponding figure caption.

**Results**

**Cytokine secretion and monocyte activation in a dynamic microfluidic environment acting as inflammation recruitment model**

Changes in the levels of the pro-inflammatory cytokine IL-6 were quantified as an indicator of an acute inflammatory response in the various in vitro models to AuNPs exposure (Figs. 1A and S6). IL-6 is known to be produced at the site of inflammation and plays a key role in the first acute phase of the inflammatory response (Gabay, 2006). No endotoxin contamination was found in the AuNPs sample (Fig. S3 in the Supporting Information); thus, IL-6 secretion levels could be directly linked to the cell interactions with the nanomaterial. Supernatants harvested from those in vitro models that showed high IL-6 production were then utilised for testing monocytes activation and adhesion under dynamic microfluidic environment. Pro-inflammatory and anti-inflammatory signals, together with other physical and chemical cues, do in fact work in a “yin and yang” mode. This makes particularly difficult, if not impossible, to use ELISA to predict which signal (pro-inflammatory or...
Figure 1. Inflammatory responses to gold nanoparticles (AuNPs) exposure. (A) Interleukin-6 (IL-6) secretion in submerged cultures grown on glass substrates, mono- and co-cultures grown on Transwell™ inserts, and in MucilAir™ models. Secretion levels were normalised to each corresponding negative control (NT) set to a value equal to 1. The in vitro models were exposed to AuNPs at a concentration of 0.06 μg/cm² for 3, 6, and 24 h. Untreated models (NT) and cell cultures exposed to lipopolysaccharides (200 ng/mL; 6 h) as a positive control were also tested for IL-6 secretion for comparison. Data are reported as mean ± standard error of the mean (n tests > 3 and n replicates = 2). The symbols (*) and (**) indicate a significant difference (P value < 0.05 and P < 0.001, respectively) as compared with the corresponding NT (two-way ANOVA followed by Bonferroni post-test). (B) Table highlighting significant differences in IL-6 secretion among the four in vitro models tested (two-way ANOVA followed by Bonferroni post-test). (C-L) Monocyte adhesion assay in a dynamic microfluidic environment. (C) Average cell attachment in uncoated or recombinant human Vascular Cell Adhesion Molecule-1 (rhVCAM-1) coated channels. THP-1 cells were incubated with media or with recombinant human Monocyte Chemoattractant Protein-1 dispersed in media (1 μg/mL) as positive control. Data are reported as mean ± standard error of the mean. Changes were not statistically significant (one-way ANOVA followed by Bonferroni multiple comparison test). (D) Average number of THP-1 cells attached to the rhVCAM-1 coated channels in response to incubation with supernatants harvested from in vitro models, untreated (NT) or exposed to AuNPs (0.06 μg/cm²; 24 h). Data are reported as mean ± standard error of the mean. The symbols (*), (**) and (***) indicates a significant increase in cell attachment (P < 0.05, 0.01, and 0.001, respectively) as compared with the corresponding NT and to THP-1 cells incubated with media and perfused through rhVCAM-1 coated channels (one-way ANOVA followed by Bonferroni multiple comparison test). (E-M) Representative brightfield microscopy images (magnification: 40× objective lens) showing adherent THP-1 cells in response to incubation with (E, F) media, (G) recombinant human Monocyte Chemoattractant Protein-1, or (H-M) supernatants harvested from cell culture models, untreated or exposed to AuNPs (0.06 μg/cm²; 24 h): (H, I) mono-cultures or (J, K) co-cultures grown on Transwell™ inserts, and (L, M) MucilAir™ models. The adherent THP-1 cells can be distinguished from flowing cells by their flattened, circular, and slightly bigger structure.
anti-inflammatory) will prevail, triggering (or not) inflammation through monocytes activation. Thus, a microfluidic set-up was optimised in our centre for this study, in order to mimic the monocytes activation and recruitment from the bloodstream to the lung epithelium in response to inflammatory signals. In in vivo conditions, monocytes circulating in the bloodstream play a key role in lung inflammation (Suzuki, Chow, & Downey, 2008): in response to an inflammogogenic stimulus, these cells in fact activate and transmigrate from the bloodstream to the site of inflammation across the blood vessel wall (Blank, Rothen-Rutishauser, & Gehr, 2007; Holt, 2005).

Transmigration begins with rolling along endothelial cells, followed by firm adhesion to the vessels walls through VCAM-1 interaction and diapedesis across the endothelium towards the harmed tissue (Gerhardt & Ley, 2015). Various bioactive molecules control monocytes activation in the lung, including pro-inflammatory cytokines and chemokines secreted by epithelial cells and acting as chemoattractants for monocytes recruitment into the tissue (Shi & Pamer, 2011; Turner, Nedjai, Hurst, & Pennington, 2014). Thus, this assay was carried out with the aim to evaluate if human monocytes (THP-1 cells) would be activated in response to the pro-inflammatory signalling molecules secreted by the AuNPs-treated in vitro models, thus providing more physiological information on the in vitro parameters in in vitro models. The assay controls confirmed the validity of the set-up developed in house (Fig. 1C,E-G). The positive control (rhMCP-1 treated THP-1 cells) showed in fact an increase (although not significant) in the number of adherent cells as compared with the untreated THP-1 cells (NT), while almost no cells adhered to the microchannels surface in the absence of rhVCAM-1 coating. rhMCP-1 is known to induce monocytes activation (Ashida, Arai, Yamasaki, & Kita, 2001; Cambien, Pomeranz, Millet, Rossi, & Schmid-Alliana, 2001).

Our data clearly evidence that IL-6 secretion levels are significantly higher for submerged mono-cultures grown on Transwell™ inserts than in models grown on glass substrates (Figs. 1B and S6). LPS (6 h) did not seem to induce any significant increase in IL-6 secretion, suggesting that a longer exposure time or higher concentration might be necessary for this molecule to trigger an inflammatory response of the in vitro models tested. Overall, submerged cell models did not show any significant IL-6 secretion at the three time points tested, as compared with NT (Fig. 1A). On the contrary, AuNPs-triggered IL-6 production was time-dependent in mono-cultures grown on Transwell™ inserts, and a significant increase in IL-6 secretion was detected following 24 h exposure. Because these two in vitro models shared the same cell type and cell seeding concentration, our results suggest that the different culturing substrates might have influenced the pro-inflammatory response of A549 cells to AuNPs. Incubation of THP-1 cells in supernatants harvested from mono-cultures grown on Transwell™ inserts and exposed to AuNPs also triggered a significant increase in monocyte adhesion in a dynamic microfluidic environment (Fig. 1D,H-I).

IL-6 basal levels in untreated cultures increased with the increasing complexity of the in vitro model tested (Fig. S6). For example, the presence of lung fibroblasts in the co-cultures induced an increase in IL-6 levels in the negative control (NT) as compared with submerged mono-cultures grown on glass or on Transwell™ inserts. Similarly, MucilAir™ models, the most complex in vitro model tested in this study, showed the highest IL-6 expression in the negative control among all in vitro cultures, with almost a four-fold increase in IL-6 production as compared with submerged cultures on glass.

In vitro complexity was inversely proportional to the ability of AuNPs to trigger inflammation. No significant increase in IL-6 secretion could in fact be detected in co-cultures grown on Transwell™ inserts or in MucilAir™ models exposed to AuNPs at any of the time-points tested (Fig. 1A,B). Similarly, incubation of THP-1 cells in supernatants isolated from MucilAir™ models exposed to AuNPs did not cause any significant increase in monocyte adhesion in the dynamic microfluidic environment, when compared with the monocytes activation induced by incubation with supernatants harvested from untreated MucilAir™ models (NT) (Fig. 1D,L-M). In contrast, supernatants collected from co-cultures grown on Transwell™ inserts (i.e., in vitro...
models formed by multiple cell types, comparable culturing substrate but a less complex structure than MucilAir™ models) triggered an enhanced monocyte activation and adhesion when the cultures were exposed to AuNPs (Fig. 1D,J–K).

**Cellular internalisation of gold nanoparticles**

Following exposure to AuNPs for 3, 6, and 24 h, in vitro models were immunostained and imaged by LSCM. Analysis of z-stack LSCM images (Figs. 2 and 3) demonstrated that AuNPs did not trigger any evident change in cell morphology and/or in the cytoskeleton organisation in any of the in vitro models tested, thus confirming that, at the concentration tested (0.06 μg/cm²), AuNPs did not cause acute cytotoxicity.

Laser Scanning Confocal Microscopy imaging and analysis (Fig. 2A), Raman spectroscopy (Fig. 2B), and dark-field microscopy (Fig. 2C) successfully

**Figure 2.** Gold nanoparticles (AuNPs) internalisation in A549 cells cultured in submerged conditions on glass substrates. (A) Representative orthogonal Laser Scanning Confocal Microscopy images of z-stack series of cell cultures untreated (NT) or exposed to AuNPs at concentration of 0.06 μg/cm² for 3, 6, and 24 h. AuNPs (indicated by arrows) were imaged in reflectance mode and are shown in white as pseudo-colour. A549 cells were stained with rhodamine phalloidin (F-actin filaments, in red), Hoechst 33342 (nuclei, in blue) and mouse anti-α tubulin Alexa 488 (tubulin microtubules, in green). Scale bars: 10 μm (63× oil objective lens, plus digital zoom). (B) 3D rendering, obtained with the BIOMAGED software, of a z-stack Laser Scanning Confocal Microscopy image showing A549 cells exposed to AuNPs for 24 h: the red arrow highlights the presence of AuNPs (probably in the form of aggregates) within the cell body. Nuclei were stained with Hoechst 33342 (in blue) and tubulin microtubules with mouse anti-α tubulin Alexa 488 (in green). (C) Raman spectra of AuNPs (light grey line), untreated submerged cultures grown on glass substrates (black line) and A549 cells cultured in submerged conditions on glass and exposed to AuNPs (0.06 μg/cm²; 24 h) (grey line). The characteristics Raman shifts of AuNPs were detected in AuNPs-treated cultures. (D) Representative dark field microscopy image of A549 cells cultured on glass in submerged conditions and exposed to AuNPs (0.06 μg/cm²; 24 h). Aggregates of AuNPs are clearly visible as bright dots in the dark background. Scale bar: 10 μm.
Figure 3. Internalisation of gold nanoparticles (AuNPs) in mono-cultures and co-cultures grown on Transwell™ inserts, and in MucilAir™ models. (A–C) Representative orthogonal Laser Scanning Confocal Microscopy images of z-stack series. Cultures were exposed to AuNPs at concentration of 0.06 μg/cm² for 3, 6, and 24 h. Images of untreated cultures (NT) are also reported for comparison. (A, C) Cell cultures were stained with rhodamine phalloidin (F-actin filaments, in red), Hoechst 33342 (nuclei, in blue) and mouse anti-α tubulin Alexa 488 (tubulin microtubules, in green). (B) Co-cultures were stained with rhodamine phalloidin (F-actin filaments, in red) and Hoechst 33342 (nuclei, in blue). A549 cells were also stained with Cell Tracker™ Green CMFDA (whole cell, in green). (A–C) AuNPs imaged in reflectance mode and shown in white pseudo-colour are indicated by arrows: (A) no AuNPs could be detected; (B) AuNPs were detected at the cells surface after 3 h exposure; (C) AuNPs were trapped on the cilia structures. Scale bars: 10 μm (63× oil objective lens, plus digital zoom).
demonstrated that AuNPs were internalised in A549 cells grown in submerged conditions on glass substrates at all the time points tested. In detail, AuNPs could be found in the cell cytoplasm, scattered among the α-tubulin filaments forming the cells cytoskeleton (Fig. 2A). As expected, untreated cells did not exhibit any significant Raman signal; whereas, strong Raman bands, associable to the characteristics Raman shifts of AuNPs, were detected in AuNPs-treated cells (Fig. 2B). Finally, dark-field microscopy (Fig. 2C) demonstrated that a large number of AuNPs were localised within the cell body.

In contrast, no evident AuNPs internalisation could be detected by LSCM imaging and analysis in monocultures and co-cultures grown on Transwell™ inserts. TEM images did not show in fact any clear indication of AuNPs internalisation in these three in vitro models. Notably, AuNPs could be seen at the cells surface in co-cultures grown on Transwell™ inserts (Figs. 3B and 4B), where up to 4 layers of cells could be distinctly recognised by TEM (Fig. 4B), confirming the successful formation of the co-culture model. AuNPs were also detected by LSCM on the top of the MucilAir™ microtissues (Figs. 3C and 4C), trapped among the cilia structures, which were clearly identifiable by LSCM and TEM.

Discussion

Lung epithelium is the first line of defence against the inhaled NPs. Currently, in vitro models of the human lung epithelium used for NPs risk assessment can vary in their complexity level: from monolayers of immortalized cell lines, through primary cells, to the revolutionary 3D-cell cultures. A detailed summary of these models can be found in a recently published review (Gordon et al., 2015). Nevertheless, the majority of the risk assessment studies on inhaled NPs is performed on submerged monocultures grown on glass or plastic substrates. Several publications have demonstrated that co-cultures of multiple cell types have a strong influence on the observed cellular responses to inhaled NPs (Blank et al., 2007; Clift et al., 2014; Lehmann et al., 2011; Muller et al., 2010; Rothen-Rutishauser, Blank, et al., 2008; Rothen-Rutishauser, Mueller, et al., 2008; Rothen-Rutishauser et al., 2007; Stoehr et al., 2015). Thus, in our case study, four different in vitro models of the lung epithelium were tested in order to quantify whether the in vitro complexity and culturing substrates can affect the detectable pulmonary inflammatory response to AuNPs. Such models were as follows: submerged cell cultures of human lung epithelial (A549) cells grown on the glass substrates; A549 cells grown on the Transwell™ membranes as mono-cultures or co-cultures with human lung fibroblasts (MRC-5 cells); and MucilAir™ models, which are reconstituted tissues derived from human donors and cultured in ALI conditions on Transwell™ inserts. MucilAir™ cultures were selected as representative models of the upper airways epithelium, while cultures of A549 cells were used to mimic the alveolar epithelium. MucilAir™ models feature a number of unique advantages over cell cultures formed by immortalized cells (such as the A549 cell line). These advantages include fully differentiated epithelium (including basal, goblet, and ciliated cells), a mucus layer covering the epithelial cells, cilia beating, functional tight junction formation, and preservation of homeostatic state up to 1 year (Huang, Wiszniewski, & Constant, 2011). Thanks to these unique features, MucilAir™ models have been successfully used to discriminate between substance with low and high absorption in humans (Reus et al., 2014), to distinguish the respiratory sensitizers from dermal sensitizers (Huang et al., 2013), and they also have been partially explored for NPs interactions studies (Beaver et al., 2009; Friek Kuper et al., 2015). MucilAir™ models are composed by primary human cells isolated from the nasal cavity, the trachea or the bronchus. Because of the physiology of the human respiratory system, however, the primary region where most inhaled NPs deposit is the alveoli (Rogueda & Traini, 2007). Unfortunately, in vitro modelling of the human alveolar epithelium is characterised by serious limitations, as primary cells sourcing and culture maintenance are extremely difficult, while human immortalized alveolar epithelial cell lines available are limited to Type-II cells. The A549 cell line belongs to such category. A549 cells are capable of forming distinct epithelial monolayers, characterised by high confluence and mucin expression; however, these cells do not express functional tight junctions (Forbes & Ehrhardt, 2005). Nevertheless, because of the limitations listed earlier regarding the in vitro modelling of the human alveolar epithelium, A549 cells are an accepted model for nanomaterials risk assessment studies (Paur et al., 2011, De Souza...
Figure 4. Detection of internalised gold nanoparticles (AuNPs) by Transmission Electron Microscopy (TEM). (A, B) Representative TEM images of ultrathin cross-sections of A549 cells cultured as (A) mono-cultures or (B) co-cultures on Transwell™ inserts. (C) Ultrathin cross-sections of MucilAir™ models imaged by TEM. (A–C) In vitro cultures were exposed to AuNPs (0.06 μg/cm²) for 24 h or left untreated (NT). Internalisation of AuNPs could not be detected in any of the in vitro models tested. Abbreviations: c, cilia; er, endoplasmic reticulum; lb, lamellar bodies; mt, mitochondria; n, nucleus. (B, C) AuNPs (indicated by red arrows) can be recognised by their geometrical shape and contrast (B) at the cells surface or (C) trapped in the cilia.
Carvalho, Daum, & Lehr, 2014). In our case study, A549 cells were also co-cultured with human fibroblasts (MRC-5 cells), in an attempt to extend the simulation of the alveolar structure to other cellular components, such as the connective tissue cells. Co-cultures composed by at least two different cell types, one of which being stromal cells, have the advantage to allow for evaluating in vivo cell-cell interactions (Miki et al., 2012). Several studies demonstrated in fact that fibroblasts do influence the sensitivity of in vitro airway culture models to stimulating or injury events (Sacco et al., 2004), drugs treatment (Jastrzebska Jedrych, Grabowska-Jadach, Chudy, Dubko, & Brzoza, 2012), and nanomaterials exposure (Chang, Chang, Hwang, & Kong, 2007; Singh, Movia, Mahfoud, Volkov, & Prinante, 2013). A cell substrate compatible with ALI culturing conditions (i.e., the Transwell inserts) was also included in our study, with the aim of evaluating the influence of such inserts on epithelial cell response to NPs.

To serve the experimental purposes, a non-toxic AuNPs dose was used in our case study. AuNPs dose was estimated based on our previous study (Movia et al., 2014) in a way that AuNPs could achieve cellular internalisation without causing acute cytotoxicity. No significant change in cell morphology, indicative of potential AuNPs cytotoxicity, could be detected in A549 cells cultured on glass and exposed to AuNPs at concentration of 0.06 μg/cm² for 24 h (Figs. 2A and S7 in the Supporting Information). Similarly, no cytoskeleton remodelling could be noticed in monocultures and co-cultures grown on Transwell™ inserts, or in MucilAir™ models, exposed to AuNPs over 24 h (Fig. 3). This confirmed that AuNPs did not trigger acute cytotoxicity at this dose.

In our case study, quantification of IL-6 secretion was used to understand whether AuNPs did induce an inflammatory response. Epithelial cells, in fact, are known to be the major source of various pro-inflammatory mediators in response to the impairment of lung epithelium homeostasis (Mohamud et al., 2014). It has been reported that pro-inflammatory mediators (e.g., Tumour Necrosis Factor-alpha, IL-6 and InterLeukin-8) are released by such cells when NPs interact with lung epithelial cells (Napierska et al., 2012). The quantification of the levels of pro-inflammatory cytokine IL-6 in response to AuNPs exposure was also accompanied by the careful investigation of the cellular internalisation of such nanomaterial overtime by various techniques. No correlation was found between IL-6 levels and AuNPs internalisation into the epithelial cell layer. In detail, where AuNPs cellular uptake was found (i.e., submerged cultures on glass), no significant IL-6 secretion could be detected; whereas, AuNPs-treated mono-cultures models grown on Transwell™ inserts were characterised by no AuNPs internalisation (Fig. 3A) but significant IL-6 secretion (Fig. 1A) following 24 h exposure. Similar results have been recently reported in a study on polyethylene glycol-coated AuNPs in a monocyte-derived dendritic cells model (Fytianos et al., 2015). Such study showed that, although a limited AuNPs cellular uptake could be observed, a significant release of pro-inflammatory mediators was induced by NPs exposure. Our data find also agreement to other scientific reports, which describe that A549 cells cultured on Transwell™ inserts in ALI conditions respond more readily to insults as compared with submerged cultures grown on glass (Frohlich et al., 2013; Lenz et al., 2013). Based on our experimental observations, we suggest that the increased sensitivity of in vitro models grown on Transwell™ inserts is imputable to the culturing substrate used and not to the ALI condition.

In parallel, it should be noted that monocytes activation was not be directly correlated to IL-6 secretion levels. Although co-culture of A549 cells with MRC-5 cells decreased the sensitivity of the epithelium, resulting in a complete loss of IL-6 secretion in response to AuNPs exposure (Fig. 1A), significant monocyte activation and adhesion could be detected after 24 h exposure (Fig. 1D). This suggested that other pro-inflammatory mediators might have been secreted by the co-culture model and in particular by its fibroblasts component. In agreement with our hypothesis, a previously published study on MRC-5 cells (as a monoculture) reported that these cells could produce various pro-inflammatory cytokines in response to AuNPs exposure (Li, Hartono, et al., 2010). Literature data show also that in a triple co-culture model grown on Transwell™ inserts and including A549 cells but not MRC-5 cells, no protein induction of pro-inflammatory cytokines (including IL-6) could be detected in response to exposure to AuNPs of comparable size (Brandenberger et al., 2010). When analysing the IL-6 secretion levels of MucilAir™ models and the monocyte activation triggered by supernatants harvested from such cultures, it was obvious once again, that in vitro complexity was inversely proportional to inflammation. Neither any significant IL-6 could be detected (Fig. 1A),
The curious case of in vitro complexity

Dania Movia et al.

nor monocyte activation (Fig. 1C), after AuNPs exposure for up to 24 h. Interestingly, in MucilAir™ models AuNPs were found trapped on the cilia structure (Figs. 3C and 4C). The lack of inflammatory response in MucilAir™ models may be linked therefore to the fact that the beating cilia present in this model together with the remaining mucus, act as a physical barrier to AuNPs penetration and interaction with epithelial cells, thus alleviating inflammation.

Finally, no correlation could be demonstrated between the lack of AuNPs internalisation and monocytes activation. In detail, in co-cultures and MucilAir™ models grown on Transwell™ membranes, neither AuNPs internalisation was detected (Fig. 3B and C), nor monocytes activation (Fig. 1C). Thus, we suggest that the monocytes activation detected when incubating THP-1 cells with supernatants harvested from mono-cultures grown on Transwell™ membranes, was not triggered by the direct contact of the immune cells with the not-internalised AuNPs still present in solution.

Conclusions
Based on our case study, we can conclude that, in vitro cell models and culturing substrates do indeed affect the AuNPs cellular internalisation and certainly influence the inflammatory response of human epithelial cells and epithelial reconstituted tissue. AuNPs internalisation was in fact achieved only in submerged cultures grown on glass substrates, and it did not correspond to the highest inflammatory response detected. Curiously, the more complex the cell model/culture was, the less intense and sensitive the inflammatory responses became. For example, no significant secretion of IL-6 was detected in supernatants harvested from AuNPs-treated co-cultures grown on Transwell™ inserts; however, significant monocytes activation was induced by the signalling molecules secreted in such models. We hypothesise that such molecules were most likely secreted by fibroblasts. On the contrary, neither IL-6 secretion nor monocyte activation could be detected when MucilAir™ models, formed by a complex, reconstituted, pseudostratified respiratory epithelium, were exposed to AuNPs.

In conclusion, we have presented a first-case report aiming at describing the in vitro parameters that should be taken into account when selecting the most appropriate testing model for inhaled NPs. Our case study suggests that the validation of in vitro models capable of integrating more cellular components and of mimicking specific aspects of the human tissue of interest is highly needed. The authors believe that further research efforts should focus on expanding the pool of in vitro models, cell lines, and exposure time-points tested, as well as on carrying out experiments evaluating the recruitment of other circulating immune cells (e.g., neutrophils) in response to AuNPs exposure and in vitro-to-in vivo correlation studies.

CONFLICT OF INTEREST
None declared.

ACKNOWLEDGMENTS
The authors would like to thank Dr. Samuel Constant (Epithelix Sàrl, Switzerland) for kindly providing the MucilAir™ models used in this study, Dr. Dimitri Scholz and Dr. Julie Kennedy (Conway Institute, University College Dublin, Ireland) for TEM samples processing and imaging, and Dr. Alan P. Bell for the technical support with He-ion microscopy imaging. This work has been partially funded by the Irish Research Council Government of Ireland Fellowship to DM, the MSC Molecular Medicine programme of Trinity College Dublin (Ireland), and the EU FP7 NANOmRG project (grant agreement 310584) towards the partial support of the work of LDC.

AUTHORS’ CONTRIBUTION
D. M., Y. V. and A. P. M. conceived this study, D. M. and A. P. M. designed the experiments and structured the paper, D. M. and R. A. performed the biological experiments, analysed the data, and carried out statistical analysis. D. M. and L. D. C. drafted the paper. J. E. M. C. synthesised the AuNBs and performed their TEM characterisation. H. M. carried out Raman spectroscopy experiments under J. S. and M. L. C. guidance. J. S. and M. L. C. contributed to reagents/materials/analysis tools. D. M., L. D. C., R. A., J. E. M. C., H. M., M. L. C., J. S., Y. V., and A. P. M. revised the paper. D. M. and A. P. M. finalised the paper.

REFERENCES
Abate, W., Alghaithy, A. A., Parton, J., Jones, K. P., and Jackson, S. K. 2010. Surfactant lipids regulate LPS-induced interleukin-8 production in A549 lung epithelial cells by inhibiting translocation of TLR4 into lipid raft domains. Journal of Lipid Research 51:334-344.
Ahmad, J., Akhter, S., Rizwanullah, M., Amin, S., Rahman, M., Ahmad, M. Z., Rizvi, M. A., Kamal, M. A., and Ahmad, F. J. 2015. Nanotechnology-based inhalation...
treatments for lung cancer: state of the art. Nanotechnology, Science and Applications 8:55-66.

Ashida, N., Arai, H., Yamasaki, N., and Kita, T. 2001. Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. The Journal of Biological Chemistry 276:16555-16560.

Ashraf, S., Pelaz, B., Del Pino, P., Carril, M., Escudero, A., Parak, W. J., Soliman, M. G., Zhang, Q., and Carrillo-Carrion, C. 2016. Gold-based nanomaterials for applications in nanomedicine. Topics in Current Chemistry 370:169-202.

Aslan, K., Lakowicz, J. R., and Geddes, C. D. 2005. Carolan, E. J., Mower, D. A., and Casale, T. B. Beaver, L. M., Stemmy, E. J., Schwartz, A. M., Damsker, J. Blank, F., Rothen-Rutishauser, B., and Gehr, P. Brandenberger, C., Rothen-Rutishauser, B., Muhlfeld, C., Frieke Kuper, C., Grollers-Mulderij, M., Forbes, B., and Ehrhardt, C. Frohlich, E., and Salar-Behzadi, S. Frohlich, E., and 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 e12091.

Clift, M. J., Endes, C., Vanhecke, D., Wick, P., Gehr, P., Schins, R. P., Petri-Fink, A., and Rothen-Rutishauser, B. 2014. A comparative study of different in vitro lung cell culture systems to assess the most beneficial tool for screening the potential adverse effects of carbon nanotubes. Toxicological Sciences 137:55-64.

De Souza Carvalho, C., Daum, N., and Lehr, C. M. 2014. Carrier interactions with the biological barriers of the lung: advanced in vitro models and challenges for pulmonary drug delivery. Advanced Drug Delivery Reviews 60:863-875.

Balasubramanian, S. K., Poh, K. W., Ong, C. N., Kreyling, W. G., Ong, W. Y., and Yu, L. E. 2013. The effect of primary particle size on biodistribution of inhaled gold nano-agglomerates. Biomaterials 34:5439-5452.

Beaver, L. M., Stemmy, E. J., Schwartz, A. M., Damsker, J. M., Constant, S. L., Ceryak, S. M., and Patierno, S. R. 2009. Lung inflammation, injury, and proliferative response after repetitive particulate hexavalent chromium exposure. Environmental Health Perspectives 117:1896-1902.

Blank, F., Rothen-Rutishauser, B., and Gehr, P. 2007. Dendritic cells and macrophages form a transepithelial network against foreign particulate antigens. American Journal of Respiratory Cell and Molecular Biology 36:669-677.

Borm, P. J., and Kreyling, W. 2004. Toxicological hazards of inhaled nanoparticles—potential implications for drug delivery. Journal of Nanoscience and Nanotechnology 4:521-531.

Braakhuis, H. M., Park, M. V., Gosen, I., De Jong, W. H., and Cassee, F. R. 2014. Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. Particle and Fibre Toxicology 11:18.

Brandenberger, C., Rothen-Rutishauser, B., Muhlfeld, C., Schmid, Ő., Ferron, G. A., Maier, K. L., Gehr, P., and Lenz, A. G. 2010. Effects and uptake of gold nanoparticles deposited at the air-liquid interface of a human epithelial airway model. Toxicology and Applied Pharmacology 242:56-65.

Bregoli, L., Movia, D., Gavigan-Imedio, J. D., Lysaght, J., Reynolds, J., and Prina-Mello, A. 2016. Nanomedicine applied to translational oncology: a future perspective on cancer treatment. Nanomedicine 12:81-103.

Cambien, B., Pomeranz, M., Millet, M. A., Rossi, B., and Schmid-Alliana, A. 2001. Signal transduction involved in MCP-1-mediated mononcytic transendothelial migration. Blood 97:359-366.

Carolan, E. J., Mower, D. A., and Casale, T. B. 1997. Cytokine-induced neutrophil transendothelial migration is dependent upon epithelial orientation. American Journal of Respiratory Cell and Molecular Biology 17:727-732.

Chang, J. S., Chang, K. L., Hwang, D. F., and Kong, Z. L. 2007. In vitro cytotoxicity of silica nanoparticles at high concentrations: studies on the effects of surface activity type of the cell line. Environmental Science & Technology 41:2064-2068.

Chen, H., Dorrigan, A., Saad, S., Hare, D. J., Cortie, M. B., and Valenzuela, S. M. 2013. In vivo study of spherical gold nanoparticles: inflammatory effects and distribution in mice. PLoS One 8 e58208.

Chen, Y. S., Hung, Y. C., Liu, I., and Huang, G. S. 2009. Assessment of the in vivo toxicity of gold nanoparticles. Nanoscale Research Letters 4:858-864.

The curious case of in vitro complexity

Choi, E. Y., Chavakis, E., Czabanka, M. A., Langer, H. F., Fraemohs, L., Economopoulou, M., Kundu, R. K., Orlandi, A., Zheng, Y. Y., Prieto, D. A., Ballantyne, C. M., Constant, S. L., Bird, W. E., Papayannopoulou, T., Gahmberg, C. G., Udey, M. C., Vajkoczy, P., Quertermous, T., Dimmelmer, S., Weber, C., and Chavakis, T. 2008. Del-1, an endogenous leukocyte-endothelial adhesion inhibitor, limits inflammatory cell recruitment. Science 322:1101-1104.

Choi, A. W., Liang, J. F., Wong, J. S., Fu, Y., Tang, N. L., and 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 e12091.

Clift, M. J., Endes, C., Vanhecke, D., Wick, P., Gehr, P., Schins, R. P., Petri-Fink, A., and Rothen-Rutishauser, B. 2014. A comparative study of different in vitro lung cell culture systems to assess the most beneficial tool for screening the potential adverse effects of carbon nanotubes. Toxicological Sciences 137:55-64.

De Souza Carvalho, C., Daum, N., and Lehr, C. M. 2014. Carrier interactions with the biological barriers of the lung: advanced in vitro models and challenges for pulmonary drug delivery. Advanced Drug Delivery Reviews 60:863-875.
Gerhardt, T., and Ley, K. 2015. Monocyte trafficking across the vessel wall. Cardiovascular Research 107:321–330.

Gordon, S., Daneshian, M., Bouwstra, J., Caloni, F., Constant, S., Davies, H., De Bruk, G., Guzman, C. A., Fabian, E., Haltner, E., Hartung, T., Hasiwa, N., Hayden, P., Kardanov, H., Khare, S., Krug, H. F., Kneuer, C., Leist, M., Lian, G., Marx, U., Metzger, M., Ott, K., Prieto, P., Roberts, M. S., Roggen, E. L., Tralau, T., Van Den Brek, C., Walles, H., and Lehr, C. M. 2015. Non-animal models of epithelial barriers (skin, intestine and lung) in research, industrial applications and regulatory toxicology. ALTEx 32:327-378.

Gosens, I., Post, J. A., De La Fonteyne, L. J., Jansen, E. H., Geus, J. W., Cassee, F. R., and De Jong, W. H. 2010. Impact of agglomeration state of nano- and submicron sized gold particles on pulmonary inflammation. Particle and Fibre Toxicology 7:37.

Hayes, A. J., and Bakand, S. 2014. Toxicological perspectives of inhaled therapeutics and nanoparticles. Expert Opinion on Drug Metabolism & Toxicology 10:933-947.

Holt, P. G. 2005. Pulmonary dendritic cells in local immunity to inert and pathogenic antigens in the respiratory tract. Proceedings of the American Thoracic Society 2:116-120.

Huang, S., Wszniewski, L., and Constant, S. 2011. The Use of In Vitro 3D Cell Models in Drug Development for Respiratory Disease: Drug Discovery and Development-Present and Future. ISBN 978-953-307-615-7, Rijeka, Croatia: InTech.

Huang, S., Wszniewski, L., Constant, S., and Roggen, E. 2013. Potential of in vitro reconstituted 3D human airway epithelia (MuciiAir) to assess respiratory sensitizers. Toxicology In Vitro 27:1151-1156.

Hussain, S., Vanoerbeek, J. A., Luyts, K., De Voogt, V., Jacobsen, N. R., Moller, P., Jensen, K. A., Vogel, U., Ladefoged, O., Loft, S., and Wallin, H. 2009. Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE/-/- mice. Particle and Fibre Toxicology 6:2.

Jain, P. K., Lee, K. S., El-Sayed, I. H., and EL-SAYED, M. A. 2016. Coded absorbed absorption and scattering properties of gold nanoparticles of different size, shape, and composition: applications in biological imaging and biomedicine. The Journal of Physical Chemistry B 110:7238-7248.

Jastrzebska Jedrych, E., Grabowska-Jadach, I., Chudy, M., Pies, J., Kruid, P., and Brzozka, Z. 2012. Multi-function microsystem for cells migration analysis and evaluation of photodynamic therapy procedure in coculture. Biomicrofluidics 6:44116.

Jud, C., Clift, M. J., Petri-Fink, A., and Rothen-Rutishauser, B. 2010. Nanomaterials and the human lung: what is known and what must be deciphered to realise their potential advantages? Swiss Medical Weekly 143:w13758.

Khan, H. A., Abdelhalim, M. A., Alhomida, A. S., and Al-Ayed, M. S. 2013. Effects of naked gold nanoparticles on proinflammatory cytokines mRNA expression in rat liver and kidney. BioMed Research International 2013:990730.

Kim, J., Chankeshwara, S. V., Thielbeer, F., Jeong, J., Donaldson, K., Bradley, M., and Cho, W. S. 2016. Surface charge determines the lung immunogenicity: a study with polystyrene nanoparticles. Nanotoxicology 10:94-101.

Kleijer, C. L., Wienc, K., van der Ven, M., Hammers, M., Van Ravenzwaay, B., and Landsiedel, R. 2012. Hazard identification of inhaled nanomaterials: making use of short-term inhalation studies. Archives of Toxicology 86:1137-1151.

Konya, V., Peinhaupt, M., and Heinemann, A. 2014. Adhesion of eosinophils to endothelial cells or substrates under flow conditions. Methods in Molecular Biology 1178:143-156.

Kreyling, W. G., Hirsch, R., and Schieh, C. 2010. Nanoparticles in the lung. Nature Biotechnology 28:1275-1276.

Kreyling, W. G., Semmler-Behneke, M., Takenaka, S., and Moller, W. 2013. Differences in the biokinetics of inhaled, nano-versus micrometer-sized particles. Accounts of Chemical Research 46:714-722.

Landsiedel, R., Sauer, U. G., Ma-Hock, L., Schnekenburger, J., and Wiemann, M. 2014. Pulmonary toxicity of nanomaterials: a critical comparison of published in vitro assays and in vivo inhalation or instillation studies. Nanomedicine (London, England) 9:2557-2585.

Lee, K. S., and El-Sayed, M. A. 2005. Dependence of the enhanced optical scattering efficiency relative to that of absorption for gold metal nanorods on aspect ratio, size, end-cap shape, and medium refractive index. The Journal of Physical Chemistry B 109:20331-20338.

Lee, K. S., and El-Sayed, M. A. 2006. Gold and silver nanoparticles in sensing and imaging: sensitivity of plasmon response to size, shape, and metal composition. The Journal of Physical Chemistry B 110:19220–19225.

Lee, K. H., Loo, E. Y., Traini, D., and Young, P. M. 2015. Nano- and micro-based inhaled drug delivery systems for targeting alveolar macrophages. Expert Opinion on Drug Delivery 12:1009-1026.

Lehmahn, A. D., Daum, N., Bur, M., Lehr, C. M., Gehr, P., and Rothen-Rutishauser, B. M. 2011. An in vitro triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier. European Journal of Pharmaceutics and Biopharmaceutics 77:398-406.

Lenz, A. G., Karg, E., Brendel, E., Hinze-Heyn, H., Maier, K. L., Eickelberg, O., Stoeger, T., and Schmid, O. 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, submersed cell-culture conditions. BioMed Research International 2013:652632.

Leong, D. T., and Ng, K. W. 2014. Probing the relevance of 3D cancer models in nanomedicine research. Advanced Drug Delivery Reviews 79:89-106.

Li, J. C., Hartono, D. L., Ong, C. N., Bay, B. H., and Yung, L. Y. 2010. Autophagy and oxidative stress associated with gold nanoparticles. Biomaterials 31:996-6003.

Li, J. J., Muralikrishnan, S., Ng, C. T., Yung, L. Y., and Bay, B. H. 2010. Nanoparticle-induced pulmonary toxicity. Experimental Biology and Medicine (Maywood, N.J.) 235:1025-1033.

Long, A., Mitchell, S., Kashanin, D., Williams, V., Prina Mello, A., Shvets, I., Kelleher, D., and Volkov, Y. 2004. A multidisciplinary approach to the study of T cell migration. Annals of the New York Academy of Sciences 1028:313-319.

Lu, X., Zhu, T., Chen, C., and Liu, Y. 2014. Right or left: the role of nanoparticles in pulmonary diseases. International Journal of Molecular Sciences 15:17577-17600.

Madd, A. K., Plummer, L. E., Carosino, C., and Pinkerton, K. E. 2014. Nanoparticles, lung injury, and the role of oxidative stress. Annual Review of Physiology 76:447-465.

Mansour, H. M., Rhee, Y. S., and Wu, X. 2009. Nanomedicine in pulmonary delivery. International Journal of Nanomedicine 4:299-319.

McIntyre, J., Verma, N. K., Smith, R. J., Moore, C., Nerl, H., Mcevoy, N., Berner, N., Mccgovern, I., Khan, U., Lyons, P., O’Neill, L., Nicol, J., and Dernberg, G. S., Byrne, H. J., Coleman, N., and Volkov, Y. 2011. A comparison of catabolic pathways induced in primary macrophages by pristine single walled carbon nanotubes and pristine graphene. RSC Advances 6:65299-65310.
The curious case of in vitro complexity

Pauluhn, J. 2003. Overview of testing methods used in inhalation toxicity: from facts to artifacts. Toxicology Letters 140-141:183-194.

Pauluhn, J., and Mohr, U. 2000. Inhalation studies in laboratory animals—current concepts and alternatives. Toxicologic Pathology 28:734-753.

Paur, H. R., Cassee, F. R., Teeuwen, J., Fissan, H., Diabate, S., Außerheide, M., Kreyleing, W. G., Hanninen, O., Kasper, G., Riediker, M., Rothen-Rutishauser, B., and Schmid, O. 2011. In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung-A dialog between aerosol science and biology. Journal of Aerosol Science 42:668-692.

Phalen, R. F., Oldham, M. J., and Wolff, R. K. 2008. The relevance of animal models for aerosol studies. Journal of Aerosol Medicine and Pulmonary Drug Delivery 21:113-124.

Reja, B., Omid, N., Kontradowitz, K., Jurida, K., Oppermann, E., Stormann, P., Werner, I., Juengel, E., Seebach, C., and Marzi, I. 2014. Decreased inflammatory responses of human lung epithelial cells after ethanol exposure are mimicked by ethyl pyruvate. Mediators of Inflammation 2014:781519.

Reus, A. A., Maas, W. J., Jansen, H. T., Constant, S., Staal, Y. C., Van Triel, J. J., and Kuper, C. F. 2014. Feasibility of a 3D human airway epithelial model to study respiratory absorption. Toxicology In Vitro 28:238-264.

Robinson, A. J., Kashanin, D., O'Dowd, F., Fitzgerald, K., Williams, V., and Walsh, G. M. 2009. Fluvastatin and lovastatin inhibit granulocyte macrophage-colony stimulating factor-stimulated human eosinophil adhesion to inter-cellular adhesion molecule-1 under flow conditions. Clinical and Experimental Allergy 39:1866-1874.

Robinson, A. J., Kashanin, D., O'Dowd, F., Williams, V., and Walsh, G. M. 2008. Montelukast inhibition of resting and GM-CSF-stimulated eosinophil adhesion to VCAM-1 under flow conditions appears independent of cysLT1R antagonism. Journal of Leukocyte Biology 83:1522-1529.

Rogueda, P. G., and Traini, D. 2007. The nanoscale in pulmonary delivery. Part 1: deposition, fate, toxicology and effects. Expert Opinion on Drug Delivery 4:595-606.

Rothen-Rutishauser, B., Blank, F., Muhlfeld, C., and Gehr, P. 2008. In vitro models of the human epithelial airway barrier to study the toxic potential of particulate matter. Expert Opinion on Drug Metabolism & Toxicology 4:1075-1089.

Rothen-Rutishauser, B., Mueller, L., Blank, F., Brandenberger, C., Muhlfeld, C., and Gehr, P. 2008. A newly developed in vitro model of the human epithelial airway barrier to study the toxic potential of nanoparticles. ALTEX 25:191-196.

Rothen-Rutishauser, B., Muhlfeld, C., Blank, F., Musso, C., and Gehr, P. 2007. Translocation of particles and inflammatory responses after exposure to fine particles and nanoparticles in an epithelial airway model. Particle and Fibre Toxicology 4:9.

Rotoli, B. M., Gatti, R., Movia, D., Bianchi, M. G., Di Cristo, L., Fruinol, S., Sonvico, F., Bergamaschi, E., Prina-Mello, A., and Bussolati, O. 2015. Identifying contact-mediated, localized toxic effects of MWCNT aggregates on epithelial monolayers: a single-cell monitoring toxicity assay. Nanotoxicology 9:230-241.

Sacco, O., Silvestri, M., Sabatini, F., Sale, R., Defilippi, A. C., and Rossi, G. A. 2004. Epithelial cells and fibroblasts: structural repair and remodelling in the airways. Paediatric Respiratory Reviews 5(Suppl A): 533-540.

Schwald, A., Murphy, F. A., Prina-Mello, A., Poland, C. A., Byrne, F., Movia, D., Glass, J. R., Dickerson, J. C., Schultz, D. A., Jeffree, C. E., Macnee, W., and Donaldson, K. 2012. The threshold length for fiber-induced acute pleural inflammation: shedding light on
The curious case of in vitro complexity

Dania Mowia et al.

The early case of in vitro complexity

The early case of in vitro complexity in asbestos-induced mesothelioma. Toxicological Sciences 128:461-470.

Schleh, C., Holtharth, U., Hirn, S., Garcia, A., Simonelli, F., Schaffer, M., Roller, M., Gibbons, N., and Kreylng, W. G. 2013. Biodistribution of inhaled gold nanoparticles in mice and the influence of surfactant protein D. Journal of Aerosol Medicine and Pulmonary Drug Delivery 26:24-30.

Shen, M. E., Abd-Elhakim, Y. M., and Al-Ayadhi, L. Y. 2015. Pancreatic response to gold nanoparticles includes decrease of oxidative stress and inflammation in autistic diabetic model. Cellular Physiology and Biochemistry 35:586-600.

Seydoux, E., Rodriguez-Lorenzo, L., Blom, R. A., Stumbles, P. A., Petri-Fink, A., Rutishauser, B. R., Blank, F. E. von Garnier, C. 2016. Pulmonary Delivery of Cationic Gold Nanoparticles Boost Antigen-Specific CD4+ T Cell Proliferation. Nanomedicine 7:1815-1826.

Shi, C., and Pamer, E. G. 2011. Monocyte recruitment during infection and inflammation. Nature Reviews. Immunology 11:762-774.

Singh, M., Movia, D., Mahfoud, O. K., Volkov, Y., and Prina-Mello, A. 2013. Silver nanowires as prospective carriers for drug delivery in cancer treatment: an in vitro biocompatibility study on lung adenocarcinoma cells and fibroblasts. European Journal of Nanomedicine 5:195-204.

Sivakumar, S. K., Kumar, S., and Santhanam, V. 2011. Monodisperse sub-10 nm gold nanoparticles by reversing the order of addition in Turkevich method—the role of chloroacetic acid. Journal of Colloid and Interface Science 361:543-547.

Spadavecchia, J., Movia, D., Moore, C., Maguire, C. M., Mostafoori, H., Casale, S., Volkov, Y., and Prina-Mello, A. 2016. Targeted polyethylene glycolyl gold nanoparticles for the treatment of pancreatic cancer: from synthesis to proof-of-concept in vitro studies. International Journal of Nanomedicine 11:791-822.

Stoehr, L. C., Endes, C., Radauer-Preiml, I., Boyles, M. S., Casals, E., Balog, S., Pesch, M., Petri-Fink, A., Rutishauser, B., Himly, M., Clift, M. J., and Duscha, A. 2015. Assessment of a panel of interleukin-8 reporter lung epithelial cell lines to monitor the pro-inflammatory response following zinc oxide nanoparticle exposure under different cell culture conditions. Particle and Fibre Toxicology 12:29.

Sudaryo, M., Vazinska, I. M., Conca, C. P., Gilliland, D., Lall, G. S., Gibb, B. F., Bonsall, D. R., Varani, L. R., Rossi, F., and Calzolai, L. 2013. Gold nanoparticles downregulate interleukin-1beta-induced pro-inflammatory responses. Small 9:472-477.

Sun, Y., Yu, F., Sun, F., and Huang, P. 2008. Adenosine antagonists promote IL-6 release in airway epithelia. Journal of Immunology 180:4173-4181.

Sung, J. C., Pulliam, B. L., and Edwards, D. A. 2007. Nanoparticles for drug delivery to the lungs. Trends in Biotechnology 25:563-570.

Sung, J. H., Ji, J. H., Park, J. D., Song, M. Y., Song, K. S., Ryu, H. R., Yoon, J. U., Jeon, K. S., Jeong, J. H., Han, B. S., Chung, Y. H., Chang, H. K., Lee, J. H., Kim, D. W., Kelman, B. J., and Yu, T. J. 2011. Subchronic inhalation toxicity of gold nanoparticles. Particle and Fibre Toxicology 8:16.

Suzuki, T., Chow, C. W., and Downey, G. P. 2008. Role of innate immune cells and their products in lung immunopathology. International Journal of Biochemistry & Cell Biology 40:1348-1361.

Tian, F., Clift, M. J., Casey, A., Del Pino, P., Pelaz, B., Conde, J., Byrne, H. J., Rutten-Rutishauser, B., Estrada, G., De La Fuente, J. M., and Stoeger, T. 2015. Investigating the role of shape on the biological impact of gold nanoparticles in vitro. Nanomedicine (London, England) 10:2643-2657.

Turner, M. D., Nedjai, B., Hurst, T., and Pennington, D. J. 2014. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochimica et Biophysica Acta-Molecular Cell Research 1843:2582-2586.

Uchiyama, M. K., Deda, D. K., Rodrigues, S. F., Drewes, C. C., Bolonheis, S. M., Kiyohara, P. K., Toledo, S. P., Colli, W., Araki, K., and Farsky, S. H. 2014. In vivo and in vitro toxicity and anti-inflammatory properties of gold nanoparticle bioconjugates to the vascular system. Toxicological Sciences 142:497-507.

Verma, N. K., Conroy, J., Lyons, P. E., Coleman, J., O’Sullivan, M. P., Kornfeld, H., Kelleher, D., and Volkov, Y. 2012. Autophagy induction by silver nanowires: a new aspect in the biocompatibility assessment of nanocomposite thin films. Toxicology and Applied Pharmacology 264:451-461.

Villiers, C., Freitas, H., Couderc, R., Villiers, M. B., and Marche, P. 2010. Analysis of the toxicity of gold nano particles on the immune system: effect on dendritic cell functions. Journal of Nanoparticle Research 12:55-60.

Walling, B. E., and Lau, G. W. 2014. Perturbation of pulmonary immune functions by carbon nanotubes and susceptibility to microbial infection. Journal of Microbiology 52:227-234.

Wu, P., Mitchell, S., and Walsh, G. M. 2005. A new antihistamine levocetirizine inhibits eosinophil adhesion to vascular cell adhesion molecule-1 under flow conditions. Clinical and Experimental Allergy 35:1073-1079.

Xie, X. H., Law, H. K. W., Wang, L. J., Li, X., Yang, X. Q., and Liu, E. M. 2009. Lipopolysaccharide induces IL-6 production in respiratory syncytial virus-infected airway epithelial cells through the toll-like receptor 4 signaling pathway. Pediatric Research 65:156-162.

Yang, W., Peters, J. I., Williams, R. O., and 3RD. 2008. Inhaled nanoparticles—a current review. International Journal of Pharmaceutics 356:239-247.

Yguerabide, J., and Yguerabide, E. E. 1998. Light-scattering submicroscopic particles as highly fluorescent analogs and their use as tracer labels in clinical and biological applications. Analytical Biochemistry 262:157-176.

Yokel, R. A., and Macphail, R. C. 2011. Engineered nanomaterials: exposures, hazards, and risk prevention. J Occup Med Toxicol 6:7.

Zarogoulidis, P., Giraleli, C., and Karamanos, N. K. 2012. Inhaled chemotherapy in lung cancer: safety concerns of nanocomplexes delivered. Therapeutic Delivery 3:1021-1023.

Zhang, Y. S., and Khademhosseini, A. 2015. Seeking the right context for evaluating nanomedicine: from tissue models in petri dishes to microfluidic organs-on-a-chip. Nanomedicine (London, England) 10:685-688.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Schematics of the in vitro cell culture models used in our study: (A) submerged cell cultures of human lung epithelial (A549) cells grown on the glass used in our study: (A) submerged cell cultures of human lung epithelial (A549) cells grown on the glass substrates; (C, D) A549 cells grown on Transwell™ inserts (schematically represented in B) as (C) monor (D) co-cultures with human lung fibroblasts (MRC-5 cells); and (E) MucliAir™ models, which are reconstituted tissues derived from human donors and cultured in ALI conditions on Transwell™ inserts. (A-E) Please note that drawings are not in scale.
Figure S2. AuNPs characterization by Transmission Electron Microscopy (TEM). The specimen was prepared by dropping an aliquot (10 μL c.a.) of the AuNPs dispersion in water (5.4×10⁻⁹ M) on 200 mesh Cu holey carbon grids and incubating for some minutes. The grids were then drained, dried for 1 day and visualized under a Jeol 2100 (Jeol 2100, Tokyo, Japan) operating at 200 kV with a Lanthanum Hexaborise emission source. (A) Representative TEM image of the AuNPs used in this study. Scale bar: 25 nm. (B) Size distributions graph based on TEM images. The average size value was calculated on a sample population of 100 AuNPs. Mean diameter (ØTEM) = 12.5 ± 1.0 nm.

Figure S3. Endotoxin units (EU) detected in AuNPs dispersion in water by means of Pierce® LAL Chromogenic Endotoxin Quantitation kit (Thermo Scientific, Ireland). No endotoxin contamination could be detected in the AuNPs sample. The AuNPs sample was tested at concentration comparable to that tested in vitro models. The assay was carried out as for manufacturer’s protocol, and absorbance was measured at 405 nm to detect the yellow chromogenic color developed in the presence of endotoxin. Dilutions of standards and AuNPs were prepared in endotoxin-free water. The AuNPs sample was tested with and without (blank) the presence of LAL chromogenic substrate to determine any inference in the assay due to the optical properties of the nanomaterial itself. Blank values were subtracted from the readings of the AuNPs sample to accurately determine the endotoxin contamination. Results are reported as average (nreplicates = 2) ± standard deviation. Lower limit of detection of the assay = 0.1 EU/ml; upper limit of detection = 1 EU/ml, as declared by manufacturer.

Figure S4. Representative set of data showing the changes in IL-6 secretion by untreated (NT) or AuNPs-treated co-cultures grown on Transwell™ inserts. IL-6 content was quantified in both apical and basolateral compartments of the cultures. While a significant production of such pro-inflammatory cytokine could be detected in the apical compartment of the cultures tested, no significant amount of IL-6 were found in the basolateral compartment. Cell cultures were exposed to AuNPs (0.06 μg/cm²) for 3, 6 and 24 h. Data are reported as mean ± standard deviation.

Figure S5. Representative calibration curve deriving from an IL-6 standard dissolved in assay diluent with (in blue; r² = 0.99) or without (in black; r² = 0.98) the addition of the AuNPs dispersion (13 pM). Quenching of the absorbance signal is evident in the presence of AuNPs.

Figure S6. IL-6 secretion in submerged cultures grown on glass substrates, mono- and co-cultures grown on Transwell™ inserts, and in MucilAir™ models. Secretion levels are not normalized. The in vitro models were exposed to AuNPs at a concentration of 0.06 μg/cm² for 3, 6 and 24 h. Untreated models (negative control or NT) and cell cultures exposed to LPS (200 ng/ml; 6 h) as a positive control (PT) were also tested for IL-6 secretion for comparison. Data are reported as mean ± standard error of the mean (n = 3 and nreplicates = 2). The symbols (⁎) and (****) indicate a significant difference (p value < 0.05 and p < 0.001, respectively) as compared to the corresponding NT (two-way ANOVA followed by Bonferroni post-test).

Figure S7. Representative Helium Ion Microscopy (HIM) images of A549 cells cultured on the glass substrates in submerged conditions and imaged by a Zeiss Orion Plus He-ion microscope (Carl Zeiss, Oberkochen, Germany): (A) untreated (NT) or (B-C) exposed to AuNPs at concentration equal to 0.06 μg/cm² for 24 h. Specimens were prepared and imaged as previously described (Movia et al., 2014). Scale bars: (A-B) 10 μm; (C) 5 μm.

Video S1. Video of a representative region of a MucilAir™ culture, showing the characteristic cilia beating (40x objective lens; 360 frames; 8 ms interval).

Video S2. Representative video of THP-1 cells flowing into uncoated channels (flow direction: right to left; 20x objective lens; 360 frames; 8 ms interval). No significant monocyte adhesion to the substrate can be noticed.

Video S3. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after incubation for 30 min in media (flow direction: right to left; 20x objective lens; 360 frames; 8 ms interval).

Video S4. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after exposure to rhMCP-1 (flow direction: right to left; 20x objective lens; 360 frames; 8 ms interval). Some monocytes adhere to the channel substrate, demonstrating activation.

Video S5. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after 30 min incubation in media harvested from untreated (NT) mono-cultures grown on Transwell™ inserts (flow direction: right to left; 20x objective lens; 360 frames; 8 ms interval).
Video S6. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after 30 min incubation in media harvested from mono-cultures grown on Transwell™ inserts exposed to AuNPs for 24 h (flow direction: right to left; 20× objective lens; 360 frames; 8 ms interval). Monocytes activation is evident.

Video S7. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after 30 min incubation in media harvested from untreated (NT) co-cultures grown on Transwell™ inserts (flow direction: right to left; 20× objective lens; 360 frames; 8 ms interval).

Video S8. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after 30 min incubation with media harvested from co-cultures grown on Transwell™ inserts exposed to AuNPs for 24 h (flow direction: right to left; 20× objective lens; 360 frames; 8 ms interval). The activated monocytes rolling and adhering to the channel substrate can be easily distinguished.

Video S9. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after 30 min incubation with media harvested from untreated (NT) MucilAir™ cultures (flow direction: right to left; 20× objective lens; 360 frames; 8 ms interval).

Video S10. Representative video of THP-1 cells flowing into rhVCAM-1 coated channels after 30 min incubation with media harvested from MucilAir™ cultures exposed to AuNPs for 24 h (flow direction: right to left; 20× objective lens; 360 frames; 8 ms interval).