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In vitro models of the respiratory barrier: a case study on zinc oxide nanoparticles

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PREFACE

Aims, abstract and structure of the thesis

AIMS

The aim of the present research of thesis is to elucidate the effects of zinc oxide nanoparticles (nZnO) - a class of inorganic engineered nanoparticles (NPs) - by using different *in vitro* models representative of the respiratory tree. A specific model of the air-blood barrier (ABB) was developed and applied to understand the importance of the interplay among different cell types in determining biological responses after exposure to NPs. Furthermore an important issue, i.e. the importance of exposure condition on *in vitro* biological outcomes, has been addressed. A classic exposure system, with treatments dispersed in the cell culture medium, and an air liquid interface (ALI) exposure equipment were compared.

These methods were used in order to give more insights into the definition of which model, with its advantages and disadvantages, should be more preferable for specific endpoints induced by NPs. This research focused on the nZnO-induced responses on *in vitro* cultures that mimic the respiratory barrier in particular on analysing the potential of these NPs to induce cytotoxicity, inflammation and endothelial cells dysfunction.

ABSTRACT

In the last decade, NPs have brought a huge revolution in many fields of application. Their small size (diameter lower than 100 nm) consent them to have unique chemical and physical properties, which, besides their positive impact in industrial use, may induce adverse health effects too. The

production and the emission of nZnO is highly increased during the last years, for their industrial, cosmetic, anti-microbial and biomedical applications. Several *in vitro* and *in vivo* studies have shown that nZnO are toxic to different mammalian cells inducing inflammation, oxidative stress and systemic effects. However, many questions about the mechanisms of nZnO-induced toxicity are still unclear. Inhalation is the major way of entry and NPs, for their small dimension, can evade the clearance of the respiratory tree and reach the alveoli. NPs can exert their effects mainly through two routes: 1) they may translocate across the ABB and directly enter the endothelial cells; 2) they may induce the epithelial cells to release inflammatory mediators, which subsequently promote the release of endothelial dysfunction markers from the endothelium. According to the REACH regulation (No 1907/2006/EC) and the 3Rs (Refinement, Reduction, Replacement) principle (Russel and Burch, 1959), *in vitro* alternative strategies are needed to test new chemical compounds, such as NPs. Furthermore, the implementation of *in vitro* models is fundamental in order to understand the biological and molecular mechanisms triggered by a toxicant or a compound. Thus, the choice of appropriate *in vitro* models becomes crucial for the evaluation of NPs-induced effects. Co-cultures on Transwell inserts represent a useful tool for the study of NPs outcomes on different cells that cross-talk to each other. With this model, local effects on pulmonary cells and secondary effects on the overlying endothelium (such as inflammation and endothelial cells damage) effects are evaluable.

Furthermore co-cultures on inserts can be exposed at the air-liquid interface (ALI), a method of cultivation in which cells are exposed to aerosolized

particles, mimicking realistic inhalation exposure conditions found in *in vivo* systems.

STRUCTURE OF THE THESIS

The following thesis is divided in two parts.

PART I comprises two descriptive chapters aimed at giving the general and up to date background. In CHAPTER I an overview on testing materials, metal oxide nanoparticles (MONs), in particular nZnO, is presented with the aim to give a general description of their fields of application, exposure scenarios, regulatory aspects, effects at the respiratory level and mechanisms of cellular toxicity. In CHAPTER II the state of the art of the *in vitro* models used for testing NPs is presented, focusing in particular on the advantages and disadvantages of each biological model.

PART II comprises four chapters that report and discuss the results obtained during the development of the Ph.D project.

In CHAPTER III the biological effects of nZnO on cell monocultures are presented, focusing on the potential of NPs to induce cytotoxicity and cellular stress response. The human alveolar epithelial cell line NCI-H441 was used as *in vitro* model for the assessment of the potential toxicity on the respiratory system of humans exposed to nZnO.

In CHAPTER IV a graphical abstract summarizes the set-up of an *in vitro* model of the formed by human epithelial alveolar cells (NCI-H441) and endothelial cells from the micro-pulmonary circulation (HPMEC-ST.6R) co-cultured on Transwell inserts. The results discussed in this chapter have been published in the paper ‘Rossella Bengalli, Paride Mantecca, Marina Camatini, and Maurizio Gualtieri, “Effect of Nanoparticles and

Environmental Particles on a Cocultures Model of the Air-Blood Barrier," BioMed Research International, vol. 2013, Article ID 801214, 8 pages, 2013. doi:10.1155/2013/801214', in which the complete set-up and characterization of the *in vitro* ABB model is described, with also results achieved by exposing the model to particulate matter (PM_{10}) and two metal oxide nanoparticles (copper oxide and titanium dioxide) used as testing materials for the investigation of some biological endpoints (barrier integrity and release of inflammatory mediators) in the novel developed *in vitro* model.

In CHAPTER V the effects of nZnO on the ABB model are elucidated. The apical compartment (alveolar epithelial cells) of the co-culture was exposed to nZnO and the effects on barrier integrity, release of inflammatory and endothelial dysfunction mediators were evaluated, focusing the attention on the response from the endothelium. Furthermore, we also tested in a tri-culture system the importance of immune cells in determining biological responses. Monocytes cells (THP-1) were added on the basal compartment of the co-culture, and the modulation of immune cells to the biological responses induced by nZnO-exposure was investigated.

CHAPTER VI finally shows the results obtained by using two different exposure systems: submerged and air-liquid interface (ALI) cultivation were used to expose a co-culture representative of alveolar space to nZnO. The different ways of NPs administration influenced the resulting cellular response (inflammation and oxidative stress). These tests were conducted during the Ph.D project at the Comprehensive Pneumology Center (CPC), Helmholtz Zentrum of Munchen, in collaboration with Dr. Tobias Stöger and supported by MODENA Cost Action. In these experiments we used the

co-culture system in use at Helmholtz Zentrum and composed by murine alveolar macrophages (MH-S) and alveolar epithelial cell lines (LA-4) seeded on the opposite side of a Transwell insert. Supplementary data about the proposed model are given at the end of this chapter.

Finally, in CHAPTER VII – GENERAL CONCLUSIONS an overall discussion of the results from the different experiments performed is reported, pinpointing the effects induced by nZnO at the ABB, as well as the importance and the advantages of using innovative *in vitro* models for NPs testing.

PART I

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General background on *in vitro* models of the respiratory barrier to study the toxic potential of nanoparticles

Abstract

The recent ‘nanotechnological revolution’ has introduced new nanoparticles (NPs) in a wide range of consumer products. The growing production and application of NPs has resulted in an increasing emission of these particles in the environment, as well as exposure of workers and consumers, leading to a great concern on their potential adverse effects on human health and environment. Although few clinical and epidemiological evidences exist at present to associate human exposure and NPs induced adverse health outcomes, abundant toxicological data demonstrate the potential of many materials to be more dangerous when sized in nanoscale rather than in bulk or micronscale. Among nanomaterials (NMs), metal oxide nanoparticles (MONs) are an interesting and critical group, since they already constitute a significant fraction of ultrafine size emissions from industrial production and represent an at risk “element” for its impact on human health. For example worker exposure to zinc oxide (ZnO) was positively correlated with incidence of the so-called “metal fume fever”, a systemic disease characterized by pulmonary toxicity, that has been recently linked to the inhaled ZnO nanoparticles (nZnO).

Over the years the criticisms arisen about NPs, such as the emerging properties, the increasing world-market, the implementation of their application fields, the several exposure scenarios and the absence of a dedicate regulation, have posed the urgent need to improve the global knowledge about their effects on biological systems, in order to harmonize and standardize their production. Inhalation is the major route of exposure to NPs. The lungs have a series of structural and functional barriers that protect the respiratory system from inhaled particles and that prevent their

translocation into the circulation. However, despite these barriers, respiratory and cardiovascular diseases are increasing. In the last decades the production and the emission in the environment of NPs is highly increased, posing humans to unintentional exposure to these compounds. Exposure to particles with a diameter lower than 100 nm is related to the risk of developing respiratory and cardiovascular diseases. Animal tests are useful for assessing NPs systemic effects, but *in vitro* models that mimic the *in vivo* situation are needed in order to satisfy the principle of the 3Rs (Replacement, Reduction and Refinement). Furthermore, *in vitro* studies are crucial because they allow to elucidate the mechanisms underlying NPs-induced effects.

The aim of this introduction is to give an overview of the state of the art of nZnO used in this thesis and of the *in vitro* models used to study respiratory nanotoxicology. In CHAPTER I is given a general overview on NPs, with a specific paragraph dedicated to MONs characteristics, exposure scenarios and regulatory aspects, followed by a paragraph with the applications of nZnO and their potential to induce toxicity in human cells. In CHAPTER II the state of the art on the *in vitro* models used for studying the interaction of NPs with respiratory barriers is presented, focusing on the advantages and disadvantages of each methods.

CHAPTER I

Metal oxide nanoparticles: state of the art of zinc oxide nanoparticles

1. Nanoparticles: general background

The concern about NPs and NMs application in daily consumer products is highly increased over the years. NPs have attractive as well as critical properties that arise the attention on their production, application and emission in the environment.

Nowadays it is well recognized that the exposure to particulate matter (PM) present in the ambient air is associated with the increase of human health outcomes, such as respiratory and cardiovascular diseases, exacerbation of asthma and pulmonary inflammation. According to the IARC (International Agency for Research on Cancer) outdoor air pollution and PM, cause cancer in humans and assign these pollutants to the strongest category of evidence (IARC 2013; Loomis et al., 2013). It has been estimated that anthropogenic aerosol represents only 10% of the total atmospheric dust (SCENIHR/002/05, Buzea et al., 2007). Nevertheless a great concern arises about the emission of particles with diameter lower than 100 nm from the spread of NMs, having size in the nanoscale range with unknown unpredictable properties.

Nanotechnology is a science that concerns the precise manipulation and control of atomic or molecular structure of materials at a nanoscale level in order to manufacture NMs with peculiar properties and applications (Karn et al., 2005).

Nanoparticles can be distinguished by their source of production in two major categories: 1) unintentionally produced ultrafine particles (UFP), (e.g.

diesel exhaust NPs or other particles produced by combustion processes), characterized by a chemical complex nature; 2) intentionally manufactured NPs with precise chemically engineered characteristics (Oberdörster et al., 2005).

This clarification is fundamental because at the beginning of this 'nanotechnology revolution' in the scientific community there has been a great confusion about the proper definition of particles in the nanoscale range. The distinction from nano- and UFP is nowadays well defined. The International Standard Organization (ISO) proposed a pre-normative terminology to define officially NPs: 'Nanoparticles indicate nano-objects with the three external dimensions ranging from 1 to 100 nm' (ISO TS27687/2008).

Another official definition of NPs was given in 2011 by the European Commission in the Recommendation 2011/696/EU of the REACH Regulation (Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals). This recommendation defines nanomaterial as 'a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm' (2011/696/EU; OJ L 275, 20.10.2011).

Engineered NMs, thanks to their unique properties (small size, high surface/mass ratio, surface energy, charges and composition) are used for a lot of application including the fields of medicine, pharmaceutics, biotechnology, energy production, environmental sciences, electronics, clothing (Karn et al., 2005).

The market of nanotechnology is estimated to become a one trillion dollar one by 2015 (Nel et al., 2006) and it has been estimated that in the US the cost for toxicity testing of existing NPs ranges from $249 million to $1.18 billion, including long *in vivo* testing approaches (Choi et al., 2009).

Thus, there is unanimous consensus, also supported by recent research papers on estimation and measuring of nano-contamination (Mueller and Novack, 2008; Gottschalk et al., 2009) that the rapid development and use of nanotechnology will increase the risk for environment and humans to come in contact with engineered NMs, that are already included in many consumer products.

Nanotoxicology has emerged as a new discipline to investigate the potential adverse health effects of NPs. However nowadays little is known about the underlying toxicity mechanisms responsible for their toxic effects. The unique nanoscale properties are likely to affect not only the chemistry and physic but also NPs behavior in biological systems.

In vivo studies revealed that pulmonary exposure to NPs induced pulmonary inflammatory responses as well as extra pulmonary effects (Oberdörster et al., 2005; Warheit et al., 2008).

NPs can be produced from any chemical, but usually most of them are synthesized from silicon, carbon, transition metals, fullerene and metal oxides (Drobne, 2007). NPs can be divided in different classes and three big categories which have specific features have been distinguished by Ostiguy and co-workers (Ostiguy et al., 2009):

1) carbon based NPs: fullerene (C_{60}), carbon nanofibers and nanofoams, graphene and graphene nanofoils, carbon nanotubes CNT (single-walled and multi-walled) and carbon black (CB);

2) organic NPs: organic polymers, biologically inspired NPs;
3) inorganic NPs: metal nanoparticles, MONs, quantum dots (QD).

Each of these categories present specific features and are applied in different fields. Among NPs, inorganic particles are used in many areas, such as catalysis, cosmetics, optic, diagnostic and drug delivery. In particular, among inorganic NPs, MONs due to their wide use in daily consumers' products, represent a NPs group of great interest in nanotechnology, because they offer intriguing new properties for applications in future technologies (Rice et al., 2009).

2. Metal oxide nanoparticles: definitions and characteristics

MONs have a crucial role in biogeochemical processes (Wigginton et al., 2007) and they exist in nature as all well as in other natural forms. During the evolution organisms have been adapted to the presence of this compounds in the environment (Blinova et al., 2010). Concern has been arisen for manufactured metal oxides since their dimensions in the nanoscale range confer them different properties from the bulk material. Thus, for this reason they should be considered as new chemical compounds (Vippola et al., 2009).

The main MONs-specific production processes are summarized by Ostiguy et al. (2009) and can be distinguished in:

1. Chemical processes: vapour phase reactions; reactions in solid media; reaction and precipitations in liquid media; sol-gel techniques; chemical co-precipitation or hydrolysis reactions; supercritical fluids with chemical reaction

2. Physical processes: laser pyrolysis; plasma synthesis or electric arc methods; evaporation/condensation under inert or reactive partial pressure; combustion flames; supercritical fluid without chemical reaction; thermal plasma;
3. Mechanical processes: mechanical activation of powder metallurgy processes; consolidation and densification; strong deformation by torsion, lamination or friction.

MONs, that are present in the consumer products, are either added to the bulk material to reinforce the physical properties of the material or applied on the surface of the product to provide enhanced surface features (resistance, water repellency, reflectivity and photo activity). In particular NPs are present in consumers devices as sensors (Bondarenko et al., 2013). The growing production and use of such NMs result in an increasing exposure of workers and consumers, leading to a greater need for information on possible health and environmental effects.

2.1 Environmental concentrations of MONs and human health hazard

Data relative to the actual environmental concentration of MONs are still poor. Monitoring systems, which, at present, allow a realistic identification and quantification of NPs in the environment, are increasing, but still there are some gaps in this field. Thus, a precise description or a real prediction of the exposure levels for the living organisms is far away for being achieved, even if a lot of *in silico* predictive models are being developing (Cohen et al., 2012). However there are nowadays efficient instruments for the monitoring and characterization of PM, but they are often inappropriate to quantify trace concentrations of NPs, due to their small size and negligible

mass. Moreover, is quite difficult to distinguish particles of interest from the natural background even using multidisciplinary approaches with sophisticated techniques, such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), (inductively coupled plasma optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), energy dispersive X-ray analysis (EDX) (Peralta-Videa et al., 2011).

Since real exposure values are not available, predictive models are used. The main variables that can influence the predictions are the extent of the starting production volume, the phases of the NP-life cycle considered (synthesis/handling of NPs and fate of NPs containing products) and the possible transformation occurring in the environment. Usually environmental concentrations of NPs, such as titanium dioxide (TiO_2), are calculated based on probabilistic material flow analysis, which considers only the life-cycle span life of MONs containing products (Gottschalk et al., 2009). Since data on realistic exposure scenario are limited to few NPs and often derived from extrapolations, the environmental concentrations of NPs are underestimated if it is considered their exponential production and emission in the environment. Therefore it is crucial to assess NPs-related hazard and to define their possible exposure scenarios.

Direct contact with MONs can occur in indoor and outdoor environments. Occupational exposure represents a classical indoor exposure scenario and it is a serious problem since it involves the industrial scale and the research laboratories in which NPs are daily handled. The main routes of exposure are inhalation and dermal contact. Investigations about monitoring of workers exposed to NPs revealed that inhalation exposure is very limited

during production (Luther 2004), but during the powder handling, packaging and manufacturing of MONs-based materials, safety concerns are greater. Regarding the outdoor exposure scenario, NPs represent a negligible fraction in mass of the total atmospheric aerosol. Nevertheless there are several possibilities to come in contact with these materials during their whole life cycle. Environmental release of MONs can derive from intentional emissions (e.g. antimicrobial applications) or unintentional sources such as spillage, degradation of MONs-based devices, disposal waste of consumer products. Moreover, according to Gottschalk and co-workers (2011), some metal oxide materials will persist in the environment because of inefficient removal processes.

Finally yet importantly, the intentional personal exposure to MON must be considered. MONs are the main components of personal care products and cosmetic. The large-scale production of nZnO or silver nanoparticles (Ag-NP) based products, such as sunscreens, drugs and cosmetics (Mueller and Nowack 2008), is one of the main reasons of the concern about human and environmental safety.

2.2 Nanoparticles inhalation: deposition in the respiratory tract

NPs can enter into the human body through three main routes of exposure: dermal, oral and respiratory tract. For humans inhalation is the most crucial and sensitive route of exposure to airborne NPs (Becker et al. 2011; Landsiedel et al., 2010). The deeper the particles are deposited in the respiratory tree, the longer it takes to remove them from the lung and the higher the probability of adverse health effects due to particle-tissue and particle-cell interactions (Blank et al., 2009).

In order to understand the toxic potential of NPs is pivotal to know particles dosimetry, which depends on atmospheric NPs concentration, deposition rate in the airways, clearance mechanisms and subsequent retention of particles within the respiratory tract. Many dosimetry models have been developed to describe the behaviour of particles in the airways, considering the various parameters involved (Phalen, 2004; Asgharian et al., 2003).

The distribution of inhaled particles in the respiratory tree varies greatly as a function of particles size: in fact inhaled particles penetrate the respiratory system depending on mechanisms, such as inertial impact, sedimentation, diffusion, electrostatic attraction, which are related to particles size (EPA, 2004). According to Oberdörster et al. (2005) diffusion is the main mechanism for deposition of inhaled NPs in the respiratory tract.

The respiratory tree can be divided into three main regions: the nasopharyngeal (or head region), the tracheobronchial region and the alveolar or pulmonary region, where gas exchange occurs (Fig.1). Based on a predictive mathematical model (International Commission on Radiological Protection 1994) it is possible to represent the fractional deposition of inhaled particles in the nasopharyngeal (or head), tracheobronchial, and alveolar regions of the human respiratory tract under conditions of nose breathing during rest (Fig.1 from Oberdörster et al., 2005).

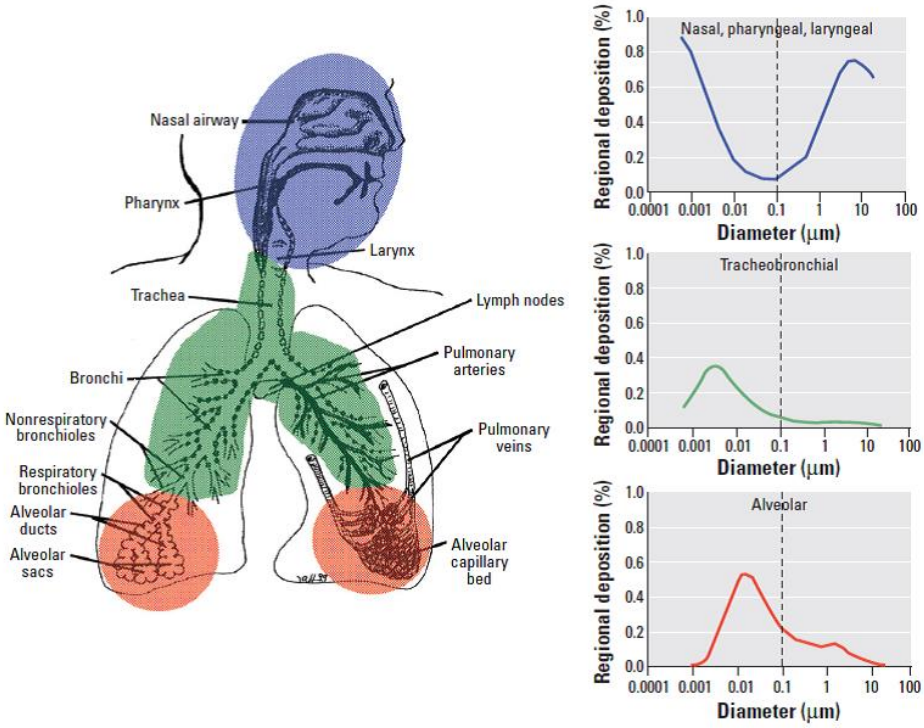

Fig. 1. The respiratory tract and predicted fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial, and alveolar region of the human respiratory tract during nose breathing.in a normal adult mouth breathing male human subject at rest. The blue line represent the nasopharyngeal (or head) region, the green one the tracheobronchial and the red one the alveolar region. (From Oberdörster et al., 2005)

Significant amounts of a certain size of NPs (1–100 nm) are deposited in each of the three regions of the respiratory tract (Fig. 1). The 90% of inhaled 1-nm particles are deposited in the head or nasopharyngeal compartment, as well as larger particles (10 μm). NPs having a size of 5nm show about equal deposition of approximately 30% of the inhaled particles in all three regions; 20-nm particles have the highest deposition efficiency in the alveolar region (~ 50%), whereas in tracheobronchial and nasopharyngeal

regions this particle size deposits with approximately 15% of efficiency (Oberdörster el al., 2005; Ostiguy et al.,2009; Geiser et al., 2010). In general particles ranging from 10-100 nm are distributed in the alveolar region. The potential effects induced by inhaled NPs could be influenced by these different deposition efficiencies.

However there are several defensive mechanisms through the respiratory tract (Kreyling and Scheuch 2000). At the alveolar region the most prevalent clearance mechanism for inhaled particles clearance is mediated by alveolar macrophages and type II pneumocytes that perform particles removal through their phagocytic activity (Hiraiwa and van Eeder 2014). The air-blood barrier (ABB), that through the formation of tight junctions (TJs) prevents the paracellular passage of the particles into the blood stream (Rothen-Rutishauseret al., 2008), constitutes another mechanism of defence at the alveolar region.

However NPs, thanks to their tiniest dimensions, may be able to escape macrophages removal, penetrate the ABB and reach other districts of the organism, where they might exert additional toxicity (Nel et al., 2006; Li et al., 2012; Cho et al., 2011).

The adverse effects of PM on human health is well established (de Kok et al., 2006; Englert, 2004; Schwarze et al., 2006) and can be divided into local effects, that rise in the lung, and systemic effects, that impact on the cardiovascular system (Donaldson and MacNee, 2001).

Current experimental toxicity evaluation of NPs, specifically carbon nanotubes, demonstrated that deposition of these materials in the lung leads to pulmonary inflammation and fibrosis (Simeonova et al., 2009).

Another study established that the accumulation of inhaled MONs in the lungs might cause systemic inflammation through oxidative stress, which mediates endothelial dysfunction and atherosclerosis (Gojova et al., 2007). In addition, NPs are also thought to increase blood coagulability through the activation of platelets leading to increased risk of cardiovascular diseases (Radomski et al., 2005).

2.3 Regulatory aspects for nanoparticles

NPs have physic-chemical properties different to their bulk counterparts, such as decreased size and increased specific surface area that enhanced reactivity, making them more efficient and interesting for different applications, but at the same time more harmful to living organisms and to human health.

In the past years the reference discipline for NPs was based on the general existing laws for chemicals and their applications to which NPs can be ascribed.

The European Commission, in the 'Regulatory Aspects of Nanomaterial', recently assessed that both NPs themselves and NPs containing products must satisfy the REACH Regulation (Registration, Evaluation, Authorization and restriction of Chemicals) criteria, since they can be generally defined as new substances or existing compounds for new applications (EC Regulation No 1907/2006).

In order to ensure the efficient assessment of manufactured nanomaterials and prevent adverse effects from the use of these materials in the short, medium and long term, the European Commission have created a specific section of the REACH specifically dedicated to NPs regulation.

In 2009 another regulatory body called OECD (the Organisation for Economic Co-operation and Development) elaborated general guidelines for testing NMs, in order to harmonize and uniform the different protocols for testing NPs (ENV/JM/MONO(2009)21). In another document a list of representative NPs 'for inclusion in a set of reference materials to support measurement, toxicology and risk' included also MONs such as silica oxide, zinc oxide, titanium dioxide (ENV/JM/MONO(2009)20/REV).

In 2012 a 'Communication on the Second Regulatory Review on Nanomaterials' described the Commission's plans to improve EU law and its application to ensure their safe use (Communication from the Commission to the European Parliament, the Council and the European Economic and Social Committee, Second Regulatory Review on Nanomaterials, Brussels, 3.10.2012, COM(2012) 572 final).

This communication states that the group of NPs that currently is attracting most attention are MONs, because even if they are marketed in smaller quantities than the traditional nanomaterials, their use is increasing fast.

This Communication is further accompanied by a 'Staff Working Paper on nanomaterial types and uses', including safety aspects, which gives a detailed overview of available information on nanomaterials on the market, including their benefits and risks. According to this communication the list of nanomaterials based on REACH registrations includes many of the substances on the OECD Working Party on Manufactured Nanomaterials (WPMN), such as MONs.

Furthermore in 2014 the OECD gave other general test guidelines about MONs genotoxicity (ENV/JM/MONO(2014)34) and about the physical

chemical properties of manufactured nanomaterials (ENV/JM/MONO(2014)15).

3. Zinc oxide nanoparticles: a case study

Zinc oxide nanoparticles (nZnO) are the third mainly produced MONs after silica (SiO_2) and titanium dioxide (TiO_2) NPs (Piccinno et al., 2012) and their production and emission in the environment are constantly increasing, thanks to their use in many fields of application, such as gas sensor, chemical sensor, bio-sensor, cosmetics, storage, optical and electrical devices, window materials for displays, solar cells, and drug-delivery (Vaseem et al., 2010).

Despite the widespread use of nZnO, the safety of this compound for humans is still unclear Furthermore, ZnO is known for its occupational hazard, since its inhalation during welding activities can lead to a systemic disease, called metal fume fever (Fine et al., 2000), characterized by enhanced inflammatory status in the lung (Kuschner et al., 1997). For these reasons we decide to focus our attention on these NPs.

3.1 Production and application of nZnO

According to different sources, the worldwide annual production of nZnO is estimated to be between 550 (Piccinno et al. 2012; Fig. 2) and 33,400 tons (Research and Markets 2012), third after SiO_2 and TiO_2 NPs (5,500 and 3,000 tons annually, respectively).

nZnO is mainly used as UV light scattering additive in cosmetics such as sunscreens, toothpastes and personal care products (Serpone et al. 2007), but encounters a larger use also in rubber manufacture, production of solar cells

and LCD disposals, pigments chemical fibres (often as whitener), electronics and textiles (Bondarenko et al. 2013). In addition, nanoscale ZnO has enhanced antibacterial properties respect to bulk material thanks to its photocatalytic activity (Padmavathy and Vijayaraghavan 2008).

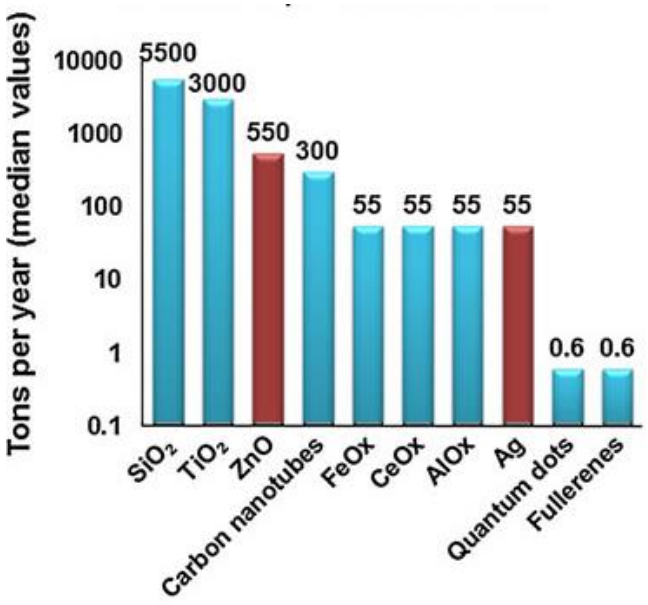

Fig 2. Annual production volumes of NMs (Bondarenko et al., 2013, data are adapted from Piccinno et al. 2012).

NPs application as antibacterials is one of the most developing field of nanotechnologies. Actually Ag-NP are the most produced NPs used to coat textiles and to confer antibacterial properties (Dastjerdi and Montazer 2010). However, there is a strong claim for an immediate regulatory police by the European Union since Ag-NPs are a very well recognized toxic agent (Foss Hansen and Baun, 2012). nZnO and copper oxide (nCuO), due to their strong redox potential that confer to them the ability to kill bacteria, seem

actually to be the best candidates in order to replace Ag-NP as anti-bacterial agents (Applerot et al., 2009; Perelshtein et al., 2009), (Lipovsky et al, 2011).

The same properties that have effects on bacteria seem to be also at the base of the mechanisms driving toxicity in human cells, and the research is still working intensely to underlie the molecular aspects involved in NPs-induced effects.

Furthermore recent works have also demonstrated that nZnO show much promise as new anticancer agents, given the specific apoptotic response of cancer cells (Akhtar et al., 2012).

Summarizing, all these fields of application will contribute to increase nZnO global production, concurrently to the likelihood to encounter humans and environment, pushing towards a better knowledge of their mechanisms of toxicity and potential adverse effects.

3.2 Potential mechanisms of nZnO toxicity

As previously said nZnO are widely used in pigments, coatings, electronic devices, but also in cosmetics, biomedical and personal care products, and the significant increase in demand, production and emissions of these NPs could lead to their unintended exposures by occupational workers and products consumers.

Experiments *in vivo* show that nZnO exposure via inhalation poses the most important hazard, while the skin represents a functional barrier against NPs (Vendebriel et al., 2012). Inhaled NPs can enter into the bloodstream after translocation through the respiratory epithelium and cause both pulmonary and cardiovascular diseases (Mühlfeld et al., 2008 ; Choi et al., 2010).

Clinical data have shown that the inhalation of nZnO cause in welders an acute adverse disease known as metal fume fever (Antonini 2003). The threshold limit values of ZnO for welders and other workers in workplaces have been set at 5 mg/m^3 (Beckett et al., 2005). There are evidences that this current exposure standard may not be adequate to protect workers from acute lung inflammation. In fact, some *in vivo* data show that metal fume fever can occur in worker exposed to 2.5 mg/m^3 for 2 hours (Fine et al., 1997). Thus, there is a strong need to better understand the mechanism behind nZnO-induced toxicity in order to prevent health hazards associated to their exposure.

In lung cells nZnO, as well as other NPs, induce several adverse effects and the most popular involved mechanisms are oxidative stress, DNA damage, inflammatory responses and cell death (Karlsson et al., 2008; Huang et al., 2010). The cell death mechanisms induced by nZnO include apoptosis, necrosis, pyroptosis and autophagy (Lai et al., 2008).

Toxicological data derived from *in vivo* models show that the inhalation of 25 mg/m^3 nZnO for 3h induces increased cytotoxicity and inflammatory proteins in the bronco-alveolar lavage fluid (BALF) of rats (Warheit et al., 2009). Zinc ions (Zn^{2+}) dissolved from nZnO can have a potential role in the inflammatory response caused by nZnO. According to Cho and coworkers (2012) the instillation of nZnO and dissolved Zn^{2+} in rat lungs caused similar pathological changes, but eosinophil recruitment was triggered only by nZnO.

In vitro cell and tissue models that mimic the cellular structure and architecture of the proximal and distal unit of the respiratory epithelium are advanced research tools for NPs toxicity screening (Klein et al., 2011).

Data from *in vitro* tests have shown that the cytotoxicity of NPs varies significantly with their size and shape (Roy et al., 2014). nZnO have greater toxic potential than bulk in human lung epithelial cells (A549) (Hanley et al., 2009; Roy et al., 2011) and rod shaped nZnO are more toxic than the spherical ones, thanks to their larger contact area with cell membrane (Yang et al., 2010).

A common mechanism for cell damage is oxidative stress. Several studies have demonstrated that NPs are able to induce reactive oxygen species (ROS) formation both *in vivo* and in *in vitro* experiments (Zhou et al., 2003, Nel et al., 2006). nZnO induce also the depletion of some ROS scavengers, such as glutathione-S-hydrogenase (GSH), superoxide dismutase (SOD) and catalase. The imbalance between the ROS formation and the depletion of the antioxidant defence system may lead to a condition of oxidative stress (Sharma et al., 2009). The inverse correlation between cell viability and the ROS level proved that oxidative stress is probably a key route for NPs to induce cytotoxicity (Li et al., 2012).

It is assumed that nZnO-induced toxicity in *in vitro* systems is caused by the solubility of nZnO, resulting in free intracellular Zn^{2+}. Zn^{2+} can be released in the culture cells medium or inside the cells (Deng et al., 2009). Zinc is a component of many enzymes, transcription factors and proteins inside the cell. However the disruption of the zinc homeostasis is linked to cytotoxicity, oxidative stress and mitochondrial dysfunction (Kao et al., 2012). Nevertheless there are controversial opinions about nZnO induced toxicity by the release of Zn^{2+}.

According to some sources, nZnO-induced injury to alveolar epithelial cells can be partially due to Zn^{2+} dissolved from nZnO (Kim et al., 2010). Other

authors, on the counterpart, have demonstrated that nZnO can induce ROS production and oxidative stress, but free Zn^{2+} and metal impurity do not appear as the major contributors of ROS induction (Lin et al., 2009).

At the alveolar capillary region, beyond alveolar epithelial cells, there also are other cell types such as alveolar macrophages, endothelial cells and immune cells of the blood circulation. In a study by Zhang et al. (2012) has been found that nZnO are able to induce cytotoxicity in alveolar macrophages. Another study conducted on monocytes revealed that this cells are more sensitive to nZnO exposure with respect to other blood cells, such as lymphocytes, and that the toxicity is driven by oxidative stress (Hanley et al., 2009).

nZnO exposure induce in cells also the expression of inflammatory cytokines (IL-1β, IL-6, TNF-α, CXCL-5, CXCL-9 and CXCL-10) (Palomaki et al., 2010) and chemokines (IL-8) (Yan et al., 2014), responsible of the recruitment of neutrophils, macrophages and lymphocytes as well as the activation of these cells, able to induce immune response (Nakae et al., 2003; Romagnani, 2006). Furthermore Gojova et al. (2007) have demonstrated that direct exposure of nZnO on endothelial cells (HAECs) upregulates the expression of inflammatory cytokines such as TNF-α, intracellular adhesion molecule-1 (ICAM-1) and IL-6 (involved in neutrophils recruitment).

Recently Bai et al. (2010) have demonstrated in an *in vivo* study that exposure to nZnO resulted in oxidative damage and inflammation response in vascular/lung endothelial cells

These data on nZnO inflammatory potential can be related with previous reports (Beckett et al., 2005; Blanc et al., 1993), demonstrating that inhaled

nZnO in humans can cause flu-like illness (metal fume fever), a systemic disease characterized by increased production of inflammatory mediators.

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CHAPTER II

In vitro models of the respiratory system: an overview

1. Nanoparticles and the respiratory barrier: general background

The internal surface area of the lungs is about 150 m^2 (Gehr et al., 1978), and at every breath this area is exposed to several potential insulting agents, such as pathogens and particles. A series of structural and functional barriers protects the respiratory system against inhaled harmful agents.

The alveoli, the distal region of the respiratory tract where pulmonary cells are in contact with the micro-capillaries of the pulmonary circulation, have two main crucial roles: they allow gas exchanges between inhaled air and circulation as well as they function as barrier, the so called air-blood barrier (ABB), to avoid the passage of pathogens and particles into the circulation. These functions of the ABB are guarantee by a monolayer of alveolar epithelial cells that are strictly adherent among them thanks to tight junctions (TJs).

Several epidemiological data have revealed that PM causes adverse health effects associated with increased pulmonary and cardiovascular diseases (Schultz et al.; 2005; Pope et al., 1995). In addition, there are increasing amounts of NPs released into the environment (Paull et al., 2003). NPs found in the environment can derive from intentional emissions (e.g. antimicrobial application or contaminant removal) or from unintentional sources during all their life cycle (e.g. production, spillage, degradation of NPs-based devices, disposal waste of consumer products).

Inhalation is the main way of exposure to NPs and there are evidences that inhaled NPs are able to reach the alveoli, cross the ABB and translocate into

the blood stream (Nemmar et al., 2002; Geiser et al., 2005). NPs can also cross the cellular membranes and induce release of pro-inflammatory cytokines, production of reactive oxygen species (ROS) and DNA damage (Rothen-Rutishauser et al., 2008). Due to these adverse effects, there is a strong need to better understanding NPs related toxicity. *In vitro* cell culture provide an alternative to *in vivo* tests for assessing the effects of particles on target organs. However, in order to understand precisely the mechanisms that drive NPs toxicity it is crucial to choose the appropriate *in vitro* system that resembles to respiratory epithelium.

The necessity to develop more sophisticated and more realistic *in vitro* models is required not only because these models could be important tools to understand the biological effects of inhaled NMs, but also because there is an urgent need of alternative strategies to animal testing (Nel et al., 2013). The fields of toxicological research have been dominated for a long period by *in vivo* animal testing, but since the formulation of the 3Rs principle (Replacement, Reduction and Refinement) (Russel and Burch, 1959), there was an increasing effort focused on reducing the number of experiments involving animals with legislative actions. These efforts were also encouraged by the European legislation, which has required over the years the development of new strategies for non-animal toxicity testing alternatives.

A detailed and comprehensive Directive of the European Union (2010/63/EU), revising the first Directive on the protection of animals used for scientific purposes (86/609/EEC), was adopted on 22 September 2010 (OJ L276, 20.10.2010). This directive removes ambiguities as to the legal requirements for using alternatives and requires the Commission and

Member States to contribute to the development and validation of alternative methods at national level aimed at refining and reducing the use of animals (Anadòn et al., 2013). Another important European Regulation is the (EC) No. 1907/2006 of the European Parliament concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) ((EC) No 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL,18.12.2006). This Regulation constituted another big step toward the reduction of animal testing for new chemical compounds and its revision in July 2014 ((EU) No 900/2014, 15.07.2014) focuses specifically to the reduction of the number of animals used for experiments, whenever is possible, by requiring that an animal experiment should not be performed when an alternative method exists, and by encouraging the development and validation of alternative methods. Nowadays two *in vitro* tests for skin corrosion have gone under validation and were adopted by the European Commission for regulatory purposes (Fentem et al., 1998) and since 2002 the OECD (Organisation for Economic Co-operation and Development) has established the first *in vitro* alternative strategy for eye irritation for regulatory purpose (OECD. TG 404; OECD. TG 405). Recently also the cell-based research has made progresses with the development of bi- and tridimensional cell cultures which closely mimic cells in the human tissues (Adler et al., 2011). With regards to nanotechnology field, according to Combes and co-workers, *in vitro* methods are more suitable than *in vivo* experiments for testing complex materials, such as NPs (Combes et al., 2011). Since NPs and NPs containing products are defined as new substances or existing compounds for new applications, they must satisfy the REACH legislation (Kaluza et al., 2011)

and it is crucial to develop *in vitro* validated alternative methods to fulfil the goals asked from the European Commission.

The major uncertainty in extrapolation from *in vitro* to *in vivo* is the need to determine the ability of simple, *in vitro* cell cultures or experiments to fully reflect the complex biology found in whole organisms (Anadòn et al., 2013).

The aim of this chapter is to give an overview on the state of the art of relevant cell-based *in vitro* assays, which are under development for the evaluation of particles induced effects.

2. Cell culture models of the lung

2.1 Organization of the lung - a short overview

The respiratory tree is a highly specialized system designed to allow gas exchange between the inhaled air and the blood stream at a high profit. It is subdivided in upper (nasal cavity, pharynx, larynx) and lower (trachea, primary bronchi, bronchioles, alveolar region) respiratory tract. The surface of the respiratory tree is formed by a continuous layer of epithelial cells, which have an important role in maintaining the functionality of the lung (Proud, 2008). Furthermore this layer shows a different cells composition between the conducting zone (bronchioli) and the distal region (alveoli) in which the gas exchange takes place. More than 40 cell types have been found within the epithelia of the respiratory systems (Sorokin, 1970). The respiratory epithelium mainly consists of two types of alveolar epithelial cells (AECs), alveolar epithelial type I cells (AEICs) and type II (AEIICs) and it is highly vascularized by capillaries underneath the basal membrane (Mann-Jong et al., 2008). AEICs cells and endothelial cells (ECs) of the

capillaries are responsible of the gas exchange between air and blood, AEIICs have different functions, such as the production of surfactant or as progenitor of AEICs, but also contributes to the defense of the alveolar space. A population of resident alveolar macrophages (AMs) is also present in the interstitial space, as first defense against pathogens and inhaled particles (BéruBé et al., 2010).

AECs are involved in many activities of the lungs; one of the most important is protecting the airways from exogenous compounds. They are also considered defender of the alveolus (Fehrenbach, 2001) because they contribute to the maintenance of the respiratory epithelium/lung homeostasis, also thanks to the crosstalk that exists between the several different cells that are present at the alveolar space. The respiratory epithelium works thanks to the formation and maintenance of the ABB (Paur et al., 2011; Pinkerton et al., 1992).

2.2. Characteristics of biological barriers

The biological barriers are important components with a crucial defensive role against harmful agents, such as pathogens or toxic compounds that can alter the functionality of an epithelium or organ. Barriers are composed by epithelial and endothelial cells that separate compartments of different cellular compositions. In forming such barriers epithelial cells polarize and form intercellular junctions (Rothen-Rutishauser et al., 2008). The most apical intercellular junctions are TJs that represent the boundary between two distinct cell membrane domains. The staining of tight junctional proteins is a method used to determine if there is the formation of a junctional complex and to evaluate the integrity of the barrier. In order to

use an *in vitro* model for transport, translocation or toxicity studies, it is crucial to determine primarily the viability/integrity of the *in vitro* barrier. As first step the Trans Epithelial Electrical Resistance (TEER), which is a good requisite for the development of an intact epithelium, must be measured (Agu et al., 2011; Geys et al., 2006).

The potential difference across the epithelium, which is a measure of the tightness of the cell-cell contact within the epithelium, can be evaluated through two electrodes or with a two-chamber system, where the compartments are separated by an epithelial monolayer grown on a porous filter inserts (Kwang-Jin, 2002). The TEER result is expressed like $\Omega*cm^2$, since it is a measure of a current through an insert. For primary alveolar epithelial cultures values of > 1000 Ω cm^2 has been described and for bronchial cell lines values between 300-400 Ω cm^2 (Elbert et al., 1999; Forbes, 2000).

In addition to the functional studies, epithelial cultures can be screened for TJs or adherens junction (AJs) proteins using a variety of methods such as real-time polymerase chain reaction (RT-PCR), western blotting, immunohistochemistry or immunofluorescence.

2.3 In vitro models of the respiratory barrier

The possible approaches used for the study of the effects of inhaled particles on the respiratory tract under controlled conditions are *in vivo* experiment, *ex vivo* studies and *in vitro* cell culture systems.

One of the big advantages of *in vitro* testing is that with this system, cellular and subcellular functions, as well as molecular pathways, can be investigated. Furthermore cultured cells can be more controlled and yield

more reproducible data than the *in vivo* situation (Rothen-Rutishauser et al., 2008).

Cells used for *in vitro* systems can be primary cells, that means derived from freshly isolated tissues, or continuous cell lines. Both systems have advantages and disadvantages.

Primary cells are highly differentiated, but will start dedifferentiation when cultured *in vitro* (Hartung et al., 2002). The main limitations for primary cells cultivation are the difficult and labour intensive isolation, the lack of availability of normal human airway tissue, the limited number of cells that can be received during each isolation and a certain donor variation (Rothen-Rutishauser et al., 2008). In addition, isolates of primary cells represent a highly heterogeneous population with respect to the state of differentiation and cell types present, but this aspect is mainly a question of good isolation procedures and could be partially overcome. (Klein et al, 2013). Cell lines are homogenous and more stable and, hence, better reproducible, but show only few characteristics of differentiated cells, wherefore they often only poorly represent the *in vivo* situation (Klein et al., 2011).

However, for establishing a new assay it is often better to use cell lines instead of primary cells to avoid possible donor variations (BéruBé et al., 2009). If used properly and after adequate characterization and validation cell lines represent a sophisticated and reproducible system that can be valid alternatives to animal experiments to understand what happens *in vivo*.

2.3.1 Monocultures of the respiratory epithelium

Monoculture systems are commonly used for studying the uptake and the toxic effects of particles and they represent the classical *in vitro* models for the screening of many toxic compounds, since they allow to study a variety of biological responses (Klein et al., 2011).

The effects of chemical compound and possible translocation of particles has been widely investigated by using the immortalised cell lines Calu-3 and 16HBE14o- (Wan et al., 2000). In addition, the immortalized cell line BEAS-2B, derived from human bronchial cells has been used to study airway epithelial functions, including the toxicity of PM (Veranth et al., 2007), but they do not form TJs (Forbes, 2000). Nevertheless, these cell lines are representative of the bronchial tract and allow to better understand the effects of NPs at their deposition site, that means the alveolar space. Culture systems of the distal region of the lungs, such as epithelial type-I or II cells are preferred.

AEIICs derived from normal lung tissue differentiate from type II to type-I like cells (Elbert et al., 1999). Primary cultures of isolated AEIICs are able to form monolayers with high TEER (>1000 Ohm*cm^2) (Fuchs et al., 2002). However cell lines are often preferred because of the ease of cell culture and in order to avoid donors variations. The cell line A549 from human lung carcinoma is the most widely used *in vitro* model, also to study the effects of PM and NPs at the respiratory level (Foster et al., 1998; Moschini et al., 2013; Longhin et al., 2013; Deng et al., 2014). A549 cells have many important biological properties of alveolar, such as membrane bound inclusions that resemble lamellar bodies, distinct polarisation, tight junctions and extensive cytoplasmic extensions (Foster et al., 1998). There

are controversial results about the capability of this cell line to express tight junctions and to generate TEER. Elbert and colleagues (1999) have found that A549 cells did not express zonula occludens-1, while Rothen-Rutishauser and co-workers (2008) have shown that A549 have developed stable barrier with transepithelial electrical values between 140 and 180 $\Omega*cm^2$ from day 3 to day 12.

Another cell line representative of the alveolar epithelium is the NCI-H441 line. NCI-H441 cells derive from a human papillary adenocarcinoma, presumably of Clara-cells origin. They grow as an attached monolayer with epithelial cell characteristics and they express surfactant protein A (SP-A) and B (SP-B) (O'Reilly et al., 1988). While A549 cells are not able to form TJs, NCI-H441 cells form junctional complexes and develop a barrier with high measurable TEER values when differentiated with the glucocorticoid Dexamethasone and co-cultured with endothelial cells (Hermanns et al., 2004).

As previously described, the alveolar epithelium consists of 40 different cell types (Sorokin, 1970), thus the use of monocultures is an oversimplification of a complex system. Therefore, to have a better understanding of the cross talk between several different cell types, co-culture systems have been developed and applied as tools to predict NPs-induced effects to the respiratory system. Furthermore complexity can be increased exposing *in vitro* models to an Air-Liquid Interface (ALI) condition, which closely mimic the *in vivo* situation (Klein et al., 2011).

The use of different *in vitro* models representative of different specific regions of the respiratory barrier (e.g. alveolar space formed by AECs and AMs, ABB composed by AECs and ECs, involvement of cells from the

immune system), integrated also with different methods of particles exposures (submerged or ALI cultivation), could be helpful in order to have a better understanding of the complex biological outcomes that can occurs when NPs are inhaled and deposited into the lungs.

2.3.2 Co-cultures models of the air-blood barrier

Many *in vitro* consisting of co-cultures of different respiratory cell types have been developed in the last decade. For the lung barrier principally three different combinations of cell types have been established: a) epithelial cells with macrophages and dendritic cells (Rothen-Rutishauser et al., 2008); b) epithelial and endothelial cells (Hermanns et al., 2004); c) monocytes with epithelial, endothelial and mast cells (Klein et al., 2013).

AMs are the principal phagocytic and scavengers cells on the alveolar compartment and they are able to secrete cytokines, chemokines and ROS when exposed to particles (Ishii et al., 2005), inducing an inflammatory response. The communication of AMs with AECs through a network of cytokines and receptors, could enhances the toxic potential of particles (Mantovani et al., 2001). For this reason, *in vitro* co-cultures of AMs and AECs are one of the most used approach to study the hazard of particles at the alveolar space. Alfaro-Moreno et al. (2008) used a model with THP-1 monocytes transformed in macrophages co-cultured with A549 cells to study the effects of PM_{10} on respiratory cells, demonstrating that in a co-culture system the inflammatory potential of PM_{10} is enhanced (Alfaro-Moreno et al., 2008).

Nevertheless, since PM and NPs are capable to induce endothelial dysfunction (Ramos-Godinez et al., 2012), endothelial cells (ECs) can be

added to the mentioned cell lines in order to study second messenger-induced effects after particles exposure. The crosstalk between ECs and AECs that directly encounter the particles plays a crucial role in the systemic effects after an inflammatory reaction. Co-cultures with these characteristics are usually seeded in Transwell inserts: the NPs could be added on the apical side of the insert and secreted messengers can cross the membrane and affect endothelial cells in the lower compartment. NPs possibility to translocate across the ABB has been demonstrated (Nel et al., 2006), however only a minimal amount (1%) of exposed NPs are able to reach the blood circulation (Möller et al., 2010). The possibility of NPs to translocate *in vitro* is controversial. It seems that NPs are not able to cross micro porous Transwell membranes filters with a pore diameter of 0,4 µm, even without a cell layer on a top (Geys et al., 2006). Recently also Dekali and co-workers (2014) have demonstrated that the Transwell membranes commercially available do not allow sensitive assessment of NPs translocation. Nevertheless, a recent study have shown that in a monoculture of Calu-3 cultured on Transwell having pores of 3 µm NPs at low doses are able to cross the filter, without compromising the alveolar epithelial integrity, nor disrupting TJs at cell-cell borders (Cohen et al., 2014).

Particles not only can translocate into circulation but they can also exert they effect inducing the release of inflammatory mediators, such as cytokines and chemokines. These soluble proteins are able to enhance the inflammatory response especially in a system in which different cell lines are able to communicate between each other and the released mediators can influence the final biological outcomes (Fig. 1).

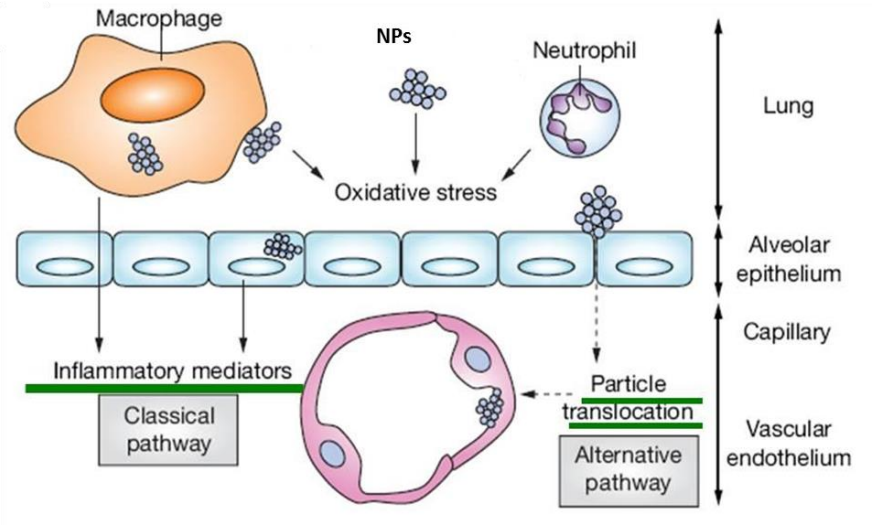

Fig. 1. Schematic representation of the major cell lines involved at the ABB level. Adapted and modified by Mills et al. 2009.

Many authors (Hermanns et al., 2004; Lison et al., 2008; Klein et al., 2013) have developed *in vitro* models of ECs and AECs co-cultured together and different types of ECs were used, such as EA.hy926, HUVEC and ISO-HAS1. However, these cell lines are not ideal for a model representative of the ABB because their origin is different from the model tissue (micro-pulmonary vasculature of the lung) (Luyts et al., 2014). In fact EA.hy926 cells are hybrid cells between HUVEC and A549, HUVEC derive from human umbilical vein endothelial cells and ISO-HAS1 cells have endothelial characteristics and derive from human mesangio-sarcoma (Klein et al., 2011).

The immortalized cell line HPMEC-ST1.6R (human pulmonary microvascular endothelial cells) could be a preferable choice since it has

pulmonary origin, micro-vascular morphology and it expresses specific endothelial phenotypic markers (Krump-Konvalinkova et al., 2001).

Although a model of the ABB composed of ECs and AECs closely mimics the *in vivo* situation, the complexity of the system can be augmented establishing a tri-culture system in which also macrophages are involved (Klein et al., 2013).

Farcal et al. (2012) have proposed a model in which THP-1 monocytes differentiated with phorbol-12-myristate-13-acetate (PMA) were added in a co-culture composed of NCI-H441 and ISO-HAS1 cells. However, they have observed a drastic decrease of TEER values after the addition of PMA, and others authors also have shown that the direct contact between PMA-activated cells and epithelial cells can change the functionality of the respiratory barrier (Lyuts et al., 2014), suggesting that in these studies a careful attention is needed in interpreting if the effects are due to the co-culture method or to NPs exposure.

In Table1 a summary of the common cells used for mono-, co- and tri-culture systems for inhalation toxicology is presented (adapted from Klein et al., 2011).

Table 1. Relevant cell types used for mono- and co-cultures *in vitro* systems.

Name	Origin	Properties	Culture system	Endpoints
Relevant cells for the respiratory epithelium				
16HBE14o -	Human bronchial epithelium	Immortalized, formation of tight junctions and cytokines expression[1]	Mono- and co-culture	Oxidative stress, inflammation, translocation of NPs[2,3]
A549	Human adeno carcinoma	Alveolar type II cells, potential to form tight junctions[4]	Mono- and co-culture	Cytotoxicity, inflammation[5,6], oxidative stress[7]
BEAS-2B	Normal human bronchial cells	Immortalized transformed by Adenovirus12-SV40 hybrid virus[8]; no tight junctions[9]; secrete cytokines	Monoculture	Cellular interaction, uptake of nanoparticles, inflammation[10]
Calu-3	Tumoral bronchial cells	Immortalised, formation of tight junctions[1]	Mono and co-culture[11]	Drug transport studies[12]; NPs translocation[11]
NCI-H441	Human carcinoma	Epithelial characteristics, expression of SP-A and SP-B[13], formation of tight junctions[14]	Mono- and cocultures	Interaction with particles, inflammation[14,15]

Name	Origin	Properties	Culture system	Endpoints
Relevant cells for the respiratory endothelium				
EA.hy 926	Hybrid between HUVEC and A549	Differentiated endothelial cells[16]	Mono- and coculture	Inflammation and cytotoxicity[17]
ISO-HAS1	Human mesangio-sarcoma	Endothelial characteristics	Mono- and coculture	Inflammation, interaction with NPs[14,18]
HPMEC-ST1.6R	Human pulmonary microvascular endothelium	Primary cells immortalized with a plasmid encoding the simian virus 40 (SV40) large T antigen; endothelial phenotype[19]	Coculture	Microvascular endothelial physiology, angiogenesis, and tumorigenesis[19], NPs translocation[11]

Name	Origin	Properties	Culture system	Endpoints
Relevant cells for the immune system				
Dendritic cells	Human peripheral blood derived	Primary cells[4]	Co-culture	Particles transport and inflammation[20]
THP-1	Human acute monocytic leukemia	Phagocytosis, potential to differentiated after different stimuli[21]	Mono- and co-culture	Inflammation, cytotoxicity[5], differentiation in alveolar macrophages to study particles interaction[22]

References: [1]Wan et al., 2000; [2]Hussain et al., 2009; [3]Hussain et al., 2010; [4]Rothen-Rutishauser et al., 2005; [5]Alfaro-Moreno et al., 2008; [6]Uboldi et al., 2009; [7]Wottrich et al., 2004;[8]Reddel et al., 1988; [9]Atsuta et al., 1997; [10]Capasso et al., 2014; [11]Dekali et al., 2014; [12]Grainger et al., 2006; [13]O'Reilly et al., 1988; [14]Hermanns et al. 2010; [15]Kasper et al., 2011; [16]Suggs et al., 1986; [17]Lison et al., 2008; [18]Farcal et al., 2012; [19]Krump-Konvalinkova et al., 2001; [20]Rothen-Rutidhauser et al., 2007; [21]Schwende et al., 1996, [22]Bengalli et al., 2013.

3. Air-Liquid Interface (ALI) exposure system

As previously said, *in vitro* cell experiments have been largely performed to assess the toxicity of NPs, as well as other toxic compounds. These studies have typically been performed using submerged exposure conditions, in which particles suspensions are directly added into the culture medium. This approach has been used also for testing airborne particles and NPs, including metal oxides, toxicity (Becker et al., 2005; Capasso et al., 2014). Although this method is widely used and it allows the fast screening of NPs effects *in vitro*, it has several criticisms for inhalation toxicology studies. For testing airborne particles submerged exposure represents an unrealistic exposure scenario, since in the *in vivo* situation the exposure occurs at the Air-Liquid Interface (ALI) (Paur et al., 2011; Blank et al., 2006). Thanks to Transwell inserts, composed of a porous membrane that is able to separate two distinct compartments, cells can be cultivated at the ALI. In this system, cells are plated on the top of the insert and the medium is added only on the basal compartment (Fig. 2).

During the ALI cultivation, cells in the apical part are in contact with the air-phase (and the aerosol containing particles) and they are not in contact with the medium. However, depending on the applied cell type, a very thin film of liquid similar to the surfactant secreted by alveolar cells can be present on the top of the cell surface/layer (Klein et al., 2011; Paur et al., 2008). It has been demonstrated that cells cultivated at the ALI, compared to the same cells in submerged conditions, are more differentiated (Yamaya et al., 1992).

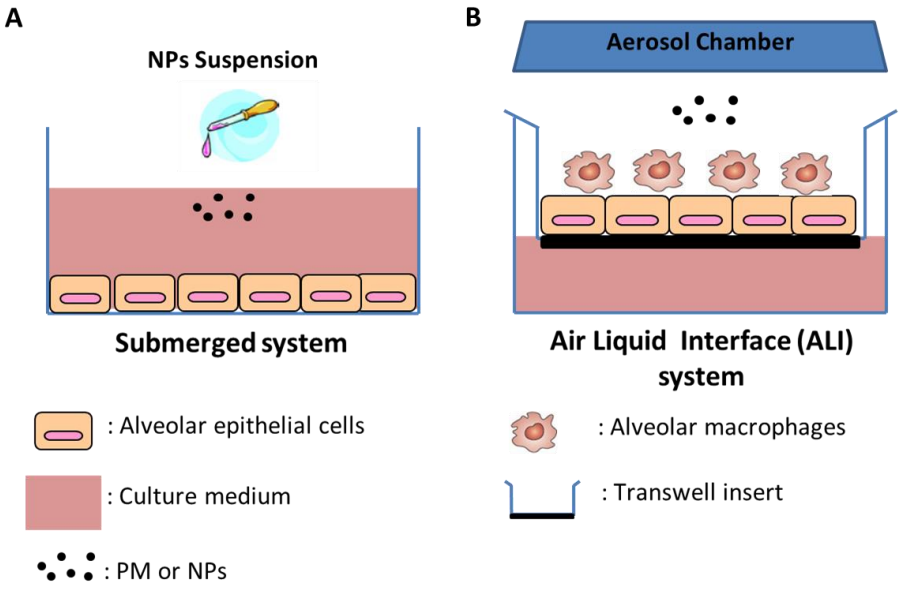

Fig. 2. Schematic representation of submerged (A) and ALI exposure (B). In figure A there is a scheme of the submerged cultivation of a monoculture of alveolar epithelial cells, in figure B a scheme of the ALI cultivation of alveolar epithelial cells co-cultured on a Transwell insert with alveolar macrophages is presented (adapted from Klein et al., 2011).

Particles can be administrated to the cells with a spraying device (e.g Microsprayer®) (Blank et al., 2006), that nebulizes the compound, or cells may be placed in an exposure chamber with an integrated aerosol generator (Lenz et al., 2009). Many other exposure devices have been developed by Vitrocell (www.vitrocell.com) and CULTEX (http://www.cultex-laboratories.com; Aufderheide and Mohr, 2000).

Another crucial benefit of ALI cultivation is the fact that particles properties are not changed by the culture medium components.

NPs status of agglomeration can be drastically modified by the medium, also for the presence of many proteins, and may result in much larger

particles than the individual NPs (Kreyling et al., 2006). In addition, the ALI exposure provides more control over the effective NPs dose interacting with the cells, which facilitates more reliable dose–response measurements. Thanks to quartz microbalance the administered dose can be continuously monitored (Paur et al., 2008; Lenz et al., 2009).

In Table 2 Submerged and Air-Liquid Interface (ALI) exposure advantages and disadvantages are summarized.

	Submerged exposure	**ALI exposure**
Advantages	+ Simple method + Fast screening + High reproducibility	+ Inhalation exposure similar to the *in vivo* situation + No alteration of particles properties by the medium + Microbalance-based dose determination
Disadvantages	- Collection of particles - Alteration of particles by the culture medium - Difficult dose-determination	- Complex and expensive

Table 2. Summary of Submerged and Air-Liquid Interface (ALI) exposure advantages and disadvantages. See the text for appropriated references.

In conclusion, ALI exposure system seems to be a promising method to study the toxicity of inhaled particles compared to the submerged condition. An important consideration should be taken into account when administrating chemicals/particles via aerosol. The greater availability of oxygen at the ALI could lead to an unintentional ROS production (Kameyama et al., 2003), and the effects caused by particles could be understimated. Nevertheless, data like the observation of significant differences of cellular response after ZnO exposure under submerged and

ALI conditions support the idea that inhalation studies should be performed under the more realistic ALI cultivation (Lenz et al., 2009). Overall the use of the co- and tri-culture systems may lead to a more realistic results regarding the hazard of new compounds in the future.

4. Commercially available models of the respiratory apparatus

Commercially cell systems of the pulmonary epithelium are available (MatTek, Epithelix). They consist of normal human bronchial cells (hAECB) and nasal cells (hAECN), primary cells derived from lung resection or biopsies from donors (Bérubé et al., 2010). These cells are able to form tight junction and they need several weeks to differentiate in a serum-free medium supplemented with growth factors and hormones (Lechner and LaVeck, 1985). To facilitate cells cultivation MatTek has developed the EpiAirway^{TM} (human tracheal or bronchial epithelium) and Epitelix has established the MucilAir^{TM} (human respiratory tract). Both models have a long lifespan and can be used for more than a year, they are useful tools for uptake studies, epithelial transport and they can be cultivated at the ALI (Balharry et al., 2008). Cells from patients suffering of cystic fibrosis, asthma, chronic obstructive disease (COPD) or from smokers are also available (www.mattek.com; www.epithelix.com).

However, these systems are made of primary cells, which have some limitations, and nowadays there are no commercially models of the alveolar region, which is the preferential site of deposition of NPs.

5. Conclusions

The evaluation of the toxic potential of new compounds, including NPs, which are nowadays present in many customer and personal care products, is an important goal. Due to the large amount of different NPs produced and released in the environment, applying *in vivo* methods may be difficult for the high number of laboratory animals needed. Thus, it is pivotal to develop sophisticated *in vitro* models to study NPs mechanisms and effects on human health. However, the majority of these *in vitro* systems are based on monocultures of cells derived from different regions of the respiratory system (Klein et al., 2011).

In vitro co-culture systems seem to be good candidates, since they have the potential to unravel some of the involved mechanisms, and could serve as a partial replacement for *in vivo* studies.

The use of cell culture based systems seems to be a major task for future toxicological studies and they can also serve as a tool to design aerosol-based inhalable drugs (Agu and Ugwoke, 2011). The increased use of these systems will further push the knowledge of mechanistic studies about NPs-induced effects. Nevertheless, a complex co-culture system, composed of different cell type and exposed at the ALI in order to mimic a more realistic situation, is still far away from resembling the broad spectrum of biological processes that occurs *in vivo*. It is important to keep in mind that the *in vitro* approaches do not consider the influence of some physical stress, such as stretching, on the epithelium. A recent study shows that physical stress can significantly alter the outcome of an *in vitro* experiment (Huh et al., 2010), thus 3-D models will be preferred (Rothen-Rutishauser et al., 2008).

In conclusion, the here presented *in vitro* systems need further development in order to properly forecast complex hazards such as respiratory inflammation and systemic effects induced by inhaled particles. The implementation into an integrated test strategy and a better knowledge about NPs effects on human health will be dependent on the cooperation of researchers from different areas (e.g. biology, physic, chemistry, epidemiology, medicine, engineering).

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PART II

CHAPTER III

Effects of zinc oxide nanoparticles on lung epithelial cells

Abstract

The use of nanomaterials (NMs) is growing exponentially and the concern about their hazard to exposed persons is highly increasing. These new materials, besides having great potential in many fields of application, also present new risks for the environment and for human health. Zinc oxide, together with titanium dioxide and silver, are the most widely used NMs. Recently, these nanoparticles (NPs) have been shown to have anti-microbial and anti-cancer effects, making them good candidates for biomedical purposes. However, it is also known that these NPs have cytotoxic effects in cells. The aim of this chapter is to focus the attention on zinc oxide nanoparticles (nZnO) potential toxic effects on epithelial lung cells, since the inhalation is the major route of exposure to particulate NMs. The effects on cell viability and on cell morphology were investigated. Furthermore, the potential of nZnO to induce ROS generation and the expression of the cellular stress markers metallothionein (MT) were also evaluated.

1. Introduction

The rapid growth in development and use of nanotechnological products increased the risk for the environment and humans to encounter NMs and consequently “nano” health safety has become an issue of major concern for the scientific and regulatory institutions. Together with carbon-based ones, metal and metal oxides (MONs) are the widest used NPs in the nanotechnology industry; NPs are globally produced in several thousand

tons per year and are widely distributed in commercial products, thus their impact on human health and environment is predicted to be increasing (Bondarenko et al., 2013). Due to the increasing production and use of NPs in variety of consumer's goods, humans are nowadays constantly exposed to such NMs (Colvin et. al, 2003). Unintended exposure to NPs may occur via inhalation, skin contact and ingestion (Stone et al., 2007). Due to their chemical and physical proprieties, NPs are able to cross the biological barriers and translocate to the blood stream, inducing vascular and systemic effects (Nemmar et al., 2002; Geiser et al., 2005; Elder et al., 2006). Furthermore, through the circulation, they could also reach secondary organs such as heart, liver, kidney and exert adverse effects. Despite NMs wide application there are still many lacking aspects for completely understanding NPs effects on human health and the mechanisms that are beyond their interaction with the cells. nZnO are one of the most commonly used group of NPs and they are used in many trades, such as industrial products, cosmetics, biomedical materials and antibacterial coating (Sahu et al, 2013). Furthermore, recent studies have shown that nZnO selectively induce apoptosis in some cancer cells (human brain tumor U87, cervical cancer HeLa and human hepatocellular carcinoma HepG2), providing preliminary guidance for the development of cancer therapy using nZnO (Wahab et al., 2013; Akhtar et al., 2012).

Nevertheless, several *in vitro* studies have also shown that nZnO are toxic to mammalian cells (Vandebriel et al., 2012). Experiments from monocultures of epithelial and bronchial cells, under submerged conditions, reported that nZnO decrease cell viability, induce oxidative stress, inflammatory response, DNA damage and cytotoxicity (Hsiao & Huang, 2011; Huang et

al., 2010; Karlsson et al., 2008; Wu et al., 2010; Xia et al., 2008). *In vivo* studies using intratracheal instillation and inhalation of ZnO NPs in rats showed lung inflammatory and cytotoxic responses (Cho et al., 2010; Sayes et al., 2007; Warheit et al., 2009). These responses resembled ‘‘metal fume fever’’ in humans – a condition associated with an increase in lung pro-inflammatory cytokines and polymorphonuclear leukocytes induced by exposures to ZnO fumes (Mihai et al., 2013). Recently, Nel and colleagues (2009) reported that cytotoxicity of nZnO may be directly related to dissolution of nZnO suspended in the culture medium of macrophages (RAW 264.7) and bronchial epithelial cell lines (BEAS-2B). Zinc is an essential element and a component of several proteins, enzymes and transcription factors. It is involved in many biological processes such as cell proliferation, differentiation and apoptosis, and it has a critical role in cellular signaling transduction (Beyersmann and Haase, 2001). An increased intracellular accumulation of this metal can lead to the production of reactive oxygen species (ROS) which can influence vital processes. Furthermore, once crossed the cellular membrane, intracellular zinc homeostasis can be regulated by metallothioneins (MT) (Tapiero and Tew, 2003), which are ubiquitous proteins with a low molecular weight (<7 kDa) and rich in cysteine-domains. MT-I and MT-II are the most diffused isoforms and they play a role in metal homeostasis via metallothionein(MT)/thionein(T) system to regulate the concentration of available zinc, copper and iron in the cell (Tapiero and Tew, 2003). MT are also known as inducible stress proteins and they can be activated by heavy metals, hormones, inflammation, oxidative stress (Andrews, 2000). They provide protection against oxidative stress and toxic metal ions (e.g. Cd, Co)

(Urani et al., 2010) reducing the biological interaction of reactive species. The expression of MT is regulated via the metal responsive element (MRE) present in the MTF-1 transcription factor. An increase of free zinc in cells serves as second messenger to activate the DNA-binding activity of MTF-1 (Smirnova et al., 2000), with consequent increase of MT expression.

As zinc-mediated MT upregulation has been found to protect against oxidative stress-induced cellular injury (Baird et al., 2006), the effect of nZnO on induction of MT expression in human epithelial alveolar cells (NCI-H441) was studied as protective mechanism.

Furthermore, since previous studies have shown that nZnO are able to interfere with the junctional complexes that occur between adjacent cells forming biological barriers, resulting in their disruption of the barrier (Kim et al., 2010), the uptake of nZnO and their capability to induce effects on tight junctions were also herewith evaluated.

2. Materials and Methods

2. 1 Preparation of NPs suspensions

nZnO (<50 nm; Sigma Aldrich, Italy) were weighted and suspended in ultrapure sterile Milli-Q water at the concentration of 8 mg/mL. Suspensions were sonicated for 10 min with the sonicator bath Ultrasonic Soniprep 150 MSE (Sanyo), vortexed for 30 seconds and then diluted in PBS + BSA (0,1% final concentration; Sigma Aldrich) in order to avoid particles agglomeration and optimize suspension stability. Working concentrations (0, 1, 5, 10, 15, 20, 25, 50 µg/mL) were obtained by adding NP suspensions directly to the culture medium (OptiMEM 1X, 1% FBS; GIBCO, Italy).

2.2 Characterization of NP suspensions

Standard, commercially available nano-sized ZnO powder from Sigma-Aldrich (#677469) was suspended in ultrapure water, sonicated, stabilized with BSA and finally diluted in culture medium (OptiMEM), added with 1%FBS, to obtain the final working NPs concentrations. The suspensions were characterized as following: the NP morphology and primary size were analysed by transmission electron microscopy (TEM); the hydrodynamic behavior and agglomeration status by dynamic light scattering (DLS) techniques.

2.2.1 Transmission electron microscopy (TEM)

The effective nZnO diameters and their size distributions were measured by TEM. nZnO were suspended in distilled water, sonicated for 1 min and vortexed for 30 seconds. Aliquots of 3 µl of the NPs suspensions (100 mg/L) were immediately pipetted and deposited onto Formvar®-coated 200 mesh copper grids, and excess of water was gently blotted using filter paper. Once dried, grids were directly inserted into a Jeol-JEM1220 TEM operating at 100 kV, and images were collected at a magnification of 50,000X using a dedicated CCD camera. More than 300 NPs of nZnO were measured, and the mean diameter (±SE) of single isolated particles was calculated for each sample.

2.2.2 Dynamic Light Scattering (DLS)

DLS was used to characterize the hydrodynamic behavior of the NPs and their extent of aggregation in suspensions in culture medium (OptiMEM 1% FBS+0,1% BSA). The light scattered at $\theta = 90°$ by the nanoparticle

suspensions was collected using a digital EMI photomultiplier (9863KB, EMI, UK) mounted on a light scattering goniometer constructed in-house (Brookhaven instruments corporation, 90 Plus, Chirico and Beretta, 1999). Data were obtained by a specific software (Brookhaven instruments corporation-90 Plus Particle Sizing Software).

2.3 Cell cultures

The human lung adenocarcinoma cell line NCI-H441 (ATCC, USA) was maintained in OptiMEM medium (Life Technologies, Italy) supplemented with 10% FBS and Pen/Strep (100 U/ml), at 37°C, 5% CO_2, split every 6 days. For cell viability experiments cells were seeded at the concentration of $2{,}8x10^5$ cells/cm^2, left in culture for 3 days and then treated with Dexamethasone (1μM) in order to induce the formation of tight barrier. The cells were treated at the 80-90% of confluence for 6h and 24h with increasing doses of nZnO (0-50 μg/ml).

2.4 Cell viability assays

MTT cytotoxicity assays were performed to screen the effects on NCI-H441 of increasing doses of nZnO (0, 1, 5, 10, 15, 20, 25, 50 μg/ml). MTT assay was performed according to Moossmann (1983), with slight modifications.
Briefly cells were rinsed with phosphate buffer saline (PBS) and MTT (final concentration 0.5 mg/ml) added for 4 h. The byproduct of MTT through the cellular metabolism (formazan crystals) was then dissolved in 1 ml DMSO. The absorbance of each sample was assessed by Multiskan Ascent (Thermo Scientific Inc.) spectrophotometer at 565 nm. The relative viability [%] related to the control samples (untreated cells) was calculated by:

Cell viability = ($OD_{sample}/OD_{control}$)x100.

The data on cell viability were reported as relative decrease compared to the control, considered as 100% of viable cells.

2.5 ROS formation

The intracellular production of reactive oxygen species (ROS) was assayed by using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Life Science Technologies, Italy). This probe detects several oxygen radicals including hydrogen peroxide, hydroxyl radicals and peroxynitrite. Cells (2.5×10^5 cells/cm^2) were treated with nZnO for 24h. After the treatment cells were washed once and incubated at 37°C with H_2DCFDA (5 μM) in PBS for 15 min, washed in PBS, harvested and suspended in PBS. The ROS induced fluorescence was quantified by flow cytometer (EPICS XL-MCL) using a 525 nm band pass filter and results analysed by dedicated software (EXPO32 ADC, Beckman–Coulter), The auto-fluorescence of the cells and nZnO was assessed analysing the signal from unstained samples (cells not incubated with H_2DCF-DA). These values were then subtracted from the values obtained from H_2DCF-DA stained samples.

2.6 Intracellular Zn^{2+} measurements

FluoZin-3 fluorescent dye (Life Technologies, Italy) was used to detect intracellular free zinc in living cells. Following exposure to nZnO (0, 10 and 20 μg/ml) or $ZnSO_4$*$7H_2O$salt (100 μM), cells were incubated with 2 μM FluoZin-3 in Opti-MEM medium (10% FBS) for 45 min at 37°C. Subsequently cells were washed with HBSS 1X (Hank's Balanced Salt

Solution, Sigma Aldrich, Italy) and incubated for additional 30 min in HBSS at 37°C. After FluoZin-3 staining the cells were trypsinized and resuspended in 500 µl HBSS for flow-cytometry measurements using EPICS XL-MCL equipped with EXPO32 ADC software (Beckman–Coulter), using a 525 nm band pass filter. Mean fluorescence values were calculated for each treatment to quantify the relative levels of free cytosolic Zn^{2+}, and results obtained from three independent experiments were normalized to control values.

2.7 Metallothionein extraction and immunoblot analysis

Cells were treated for 24h with different nZnO concentrantions (0, 10, 20 µg/ml) and $ZnSO_4*7H_2O$ (100µM) (Sigma Aldrich), used as positive control for zinc ions exposure. Stock solutions of 7mM of $ZnSO_4*7H_2O$ in ultrapure Milli Q water (Millipore) were sterilized by filtration (0.22 µm) and stored at 4°C until use. Cells grown in medium supplemented with 1% FBS+0,1% BSA represented negative control. At the end of treatments, the cells were harvested by trypsinization, collected by centrifugation (200xg, 10 min, 4°C), and washed with PBS to remove free zinc and FBS excess. The pellets obtained were processed according to Urani et al., (2010). Cell sample homogenates were prepared by resuspending the pellets in 200 ml of ice-cold 10 mM Tris-HCl (pH 7), 5 mM EDTA, 1 mM protease inhibitor PMSF and immediately frozen (-20 °C). Defrosted samples were clarified by centrifugation at 20000xg, for 45 min at 4 °C and retaining the supernatants. Total protein concentration of supernatants was determined by the BCA protein Assay (Sigma Aldrich), using BSA as a standard. The supernatants were then diluted (1:1) in sample buffer (0.25 M Tris–HCl, pH

6.8 containing 2% SDS, 30% glycerol, 10% β-mercaptoethanol, 0.01% Bromophenol blue) and stored at -20 °C until use. 20 µg of proteins were separated by SDS polyacrylamide gel electrophoresis, which was performed using 12% NuPage gels (NuPAGE® Novex 12% Bis-Tris Gel 1.0 mm, Life Technologies, Italy) at 200 V at room temperature and then processed according to Mizzen et al. (1996) to enhance membrane transfer and retention of small size proteins. Mouse anti-metallothionein (MT-I,II, Life Technologies, Italy) or anti-actin (Sigma Aldrich, Italy) were used as primary antibody. Anti-mouse alkaline phosphates conjugate (Sigma Aldrich, Italy) was used as secondary antibody and protein binding visualized by the colorimetric substrate BCIP/NBT (Sigma Aldrich, Italy) Digital images were taken by a luminescence reader (Biospectrum UVP) and densitometric analysis was performed with dedicated software (Launch VisionWorks LS). Data were normalized to the actin content and expressed as fold increase over control.

2.8 Conventional Light Microscopy

After 6h exposure to nZnO (50 µg/ml), NCI-H441 cells monolayers were fixed in 10% neutral buffer formalin and routinely stained with hematoxylin and eosin (HE). Slides were mounted in Eukitt and finally observed under Zeiss Axioplan light microscope.

2.9 Immunostaining

After nZnO exposure, NCI-H441 monolayers were washed in PBS and then fixed with 1% paraformaldehyde for 20 min at room temperature. Cells were then permeabilized with 1% Triton X-100, 1% TWEEN in PBS + 3%

BSA for 20 min at 4°C. Cells were then washed in PBS and incubated overnight at 4 °C with rabbit anti-human *zonula occludens-1* (ZO-1) antibody (1:100; Cell Signaling, Euroclone, Italy) in PBS + BSA 1% buffer. Cells were then washed three times in PBS and then incubated with the secondary antibody goat anti-rabbit Alexa Fluor-488 (1:1000, Invitrogen Molecular Probes, Italy) for 1 h. Cells nuclei were stained with Draq5 (1:250; Invitrogen Molecular Probes, Italy). Samples were mounted on a glass slide with ProLong mount (Invitrogen Molecular Probes, Italy) and observed by an AxioScope reverted microscope (Zeiss, Germany). Images were taken with the Axiovision Rel 4.8 Software (Zeiss, Germany).

2.10 Laser scanning microscopy and laser reflection

In order to visualize internalized NPs and localized them inside the cells, NCI-H441 monolayers were stained with the vital probe Draq5 (1:250; Invitrogen Molecular Probes, Italy) for the visualization of nuclei. Cells were then fixed in cold 4% paraformaldehyde, rinsed in PBS and mounted with Prolong Gold Antifade mounting medium (Invitrogen Molecular Probes, Italy). A Leica TCSNT confocal microscope was used according to the method of Prins et al. (2006) to localize NPs in laser reflection mode.

2.11 Flow cytofluorimetric analyses of NPs uptake

The extent of NPs internalization or adsorption on cell membrane was estimated by measuring forward scatter (FS) versus side scatter (SS). NCI-H441cells treated with 50µg/ml nZnO for 6h were harvested by trypsinization, resuspended in PBS and analyzed by flow cytometry

according to the methods reported in Suzuki et al. (2007). Data were analyzed using the EXPO32 ADC software (Beckman-Coulter).

2.12 Statistical analysis.

The experiments have been performed in independent triplicates, and data are reported as mean ± S.E. if not otherwise specified. Statistical comparisons have been performed with Sigmastat 3.1 software with ANOVA test and post hoc analysis. Statistical differences were considered to be significant at the 95% level ($p < 0.05$).

3. Results

3.1 nZnO characterization

According to the manufacturers (Sigma Aldrich, Italy) the size of nZnO in powder is 50 nm. TEM analysis were performed in order to determine the particles mean diameter and the particles size distribution. TEM imaging revealed particles with an irregular shape (Fig.1A) and a size distribution ranging from 10 to 40 nm and mean diameter of 38,3±0,4 nm (Fig. 1B, Table 1).

The behavior of NPs in the culture medium was assessed by DLS analysis. Once dispersed in culture medium DLS analysis showed that the global mean diameter of particles was 190,4 nm (Table1). Despite the use of BSA (0.1% final concentration) as biological stabilizing agent in NP suspensions, these results showed the tendency of nZnO to agglomerate in culture medium.

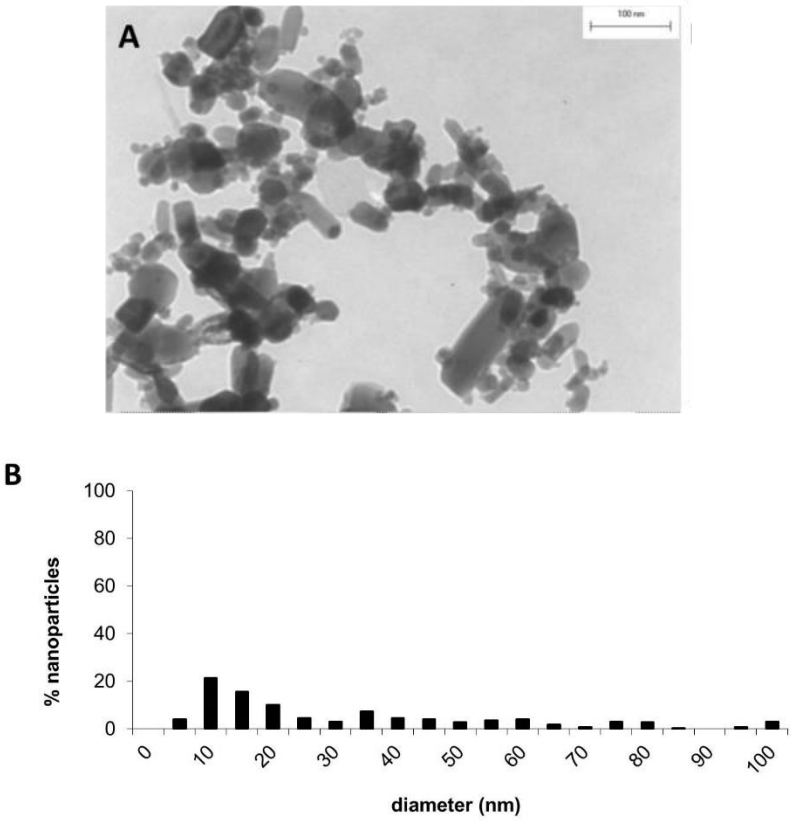


Fig.1. TEM analysis of of nZnO A) particle morphology (scale bar= 100 nm).; B) particle size distribution (% of NPs within the dimensional ranges).

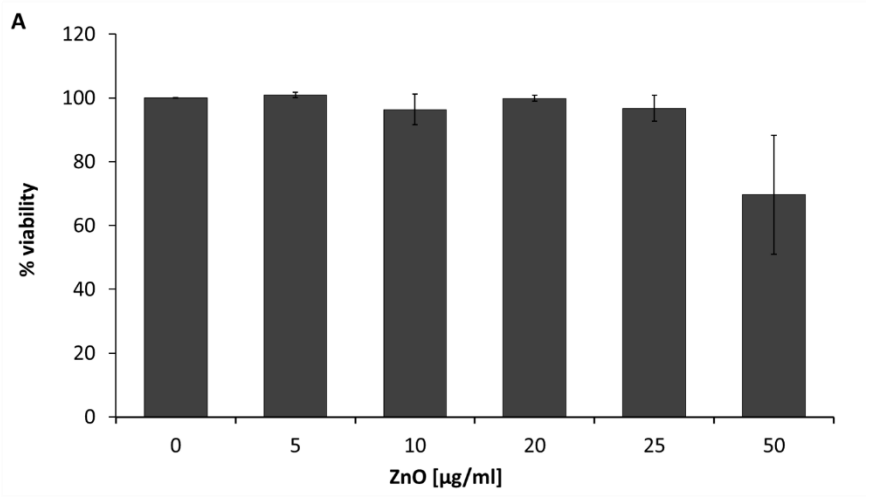
Particle	Particle size in powder[a]	Surface area[a] [m2/g]	Mean diameter in powder[b] [nm]	Average size in powder[b] [nm]	Mean diameter in solution[c] [nm]
nZnO	<50	> 10,8	38,3 ± 0,4	10-40	190,4

Table 1. nZnO description and physical chemical characterization. [a]according to the manufacturer Sigma Aldrich; [b] measured by TEM; [c] measured by DLS in culture medium.

3.2 Cytotoxicity of nZnO

The cytotoxic effects of nZnO on lung epithelial cells were assessed by the evaluation cell viability after exposure to NPs. Cells were exposed at increasing doses of nZnO choosing a range of concentrations that comprises low, intermediate and high doses of NPs, according also to literature

At 6h of exposure cell viability was not significantly affected at the maximum dose of 50µg/ml (Fig. 2A). NCI-H441 cells showed a significant ($p<0,001$) reduction of cell viability (20% and 47%) after 24h of exposure to 20 µg/ml and 25 µg/ml of nZnO, respectively (Fig. 2B). Cell viability drastically decreased after exposure to 50µg/ml of nZnO for 24h.



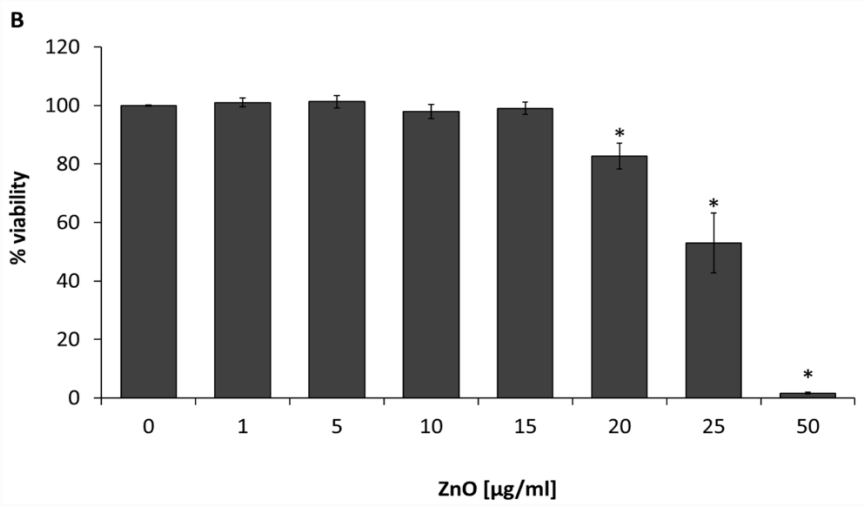


Fig. 2. Effects of nZnO on NCI-H441 cell viability. Cells viability assay (MTT assay) in NCI-H441 cells exposed to nZnO from 1 to 50 µg/ml for 6h (A) and 24h (B). The results are representative of at least three independent experiments and presented as mean ± SE. *Statistically different from control (ANOVA, Dunnett's, $p<0.001$).

3.3 ROS formation

The 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorescent probe is commonly used to detect ROS since it may react with several oxygen radicals including hydrogen peroxide, hydroxyl radicals and peroxynitrite. The cell-permeant H_2DCFDA passively diffuses into cells and it is retained into the cells after cleavage by intracellular esterases. Upon oxidation by ROS, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF), which is easily detectable (Negre-Salvayre et al., 2002).

For this experiment NCI-H441 cells were treated with 10 µg/ml and 20 µg/ml of nZnO, in order to evaluate the potential of nZnO to induce ROS

production at a non-effect dose (10 µg/ml) and at the lower-effect dose (20 µg/ml).

The quantitative measurement of intracellular ROS using H_2DCFDA-fluorescence was investigated by flow cytometry and expressed as fluorescence arbitrary units. The results are reported as fold increase over control.

NCI-H441 cells exposed to nZnO showed a significant ($p<0,05$) increase of H_2DCFDA-fluorescence intensity (2,5 fold) after the exposure to 20 µg/ml of nZnO for 24h, demonstrating the potency of nZnO to increase the level of ROS (Fig. 3).

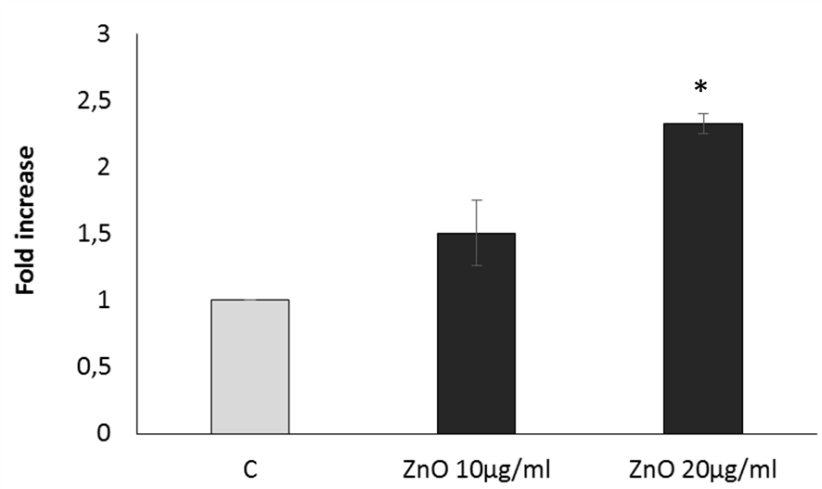


Fig. 3. ROS production evidenced by flow cytometry analysis after 24h of nZnO exposure. The data are presented as fold increase over control and represent the mean ± SE of three independent experiments. * Statistically different from control (ANOVA; $p<0,05$).

3.4 Intracellular free Zn^{2+}: FluoZin-3

In human lung epithelial NCI-H441 cells, nZnO treatment resulted in elevation of intracellular Zn^{2+} in a dose-dependent manner (Fig. 4), as demonstrated by the increase of the highly Zn^{2+}-sensitive dye FluoZin-3 fluorescence. The quantitative measurement of intracellular Zn^{2+} using FluoZin-3 fluorescence was investigated by flow cytometry and expressed as fluorescence arbitrary units. The results are reported as fold increase over control.

At 20µg/ml intracellular Zn^{2+} increased 4-fold respect to the control.

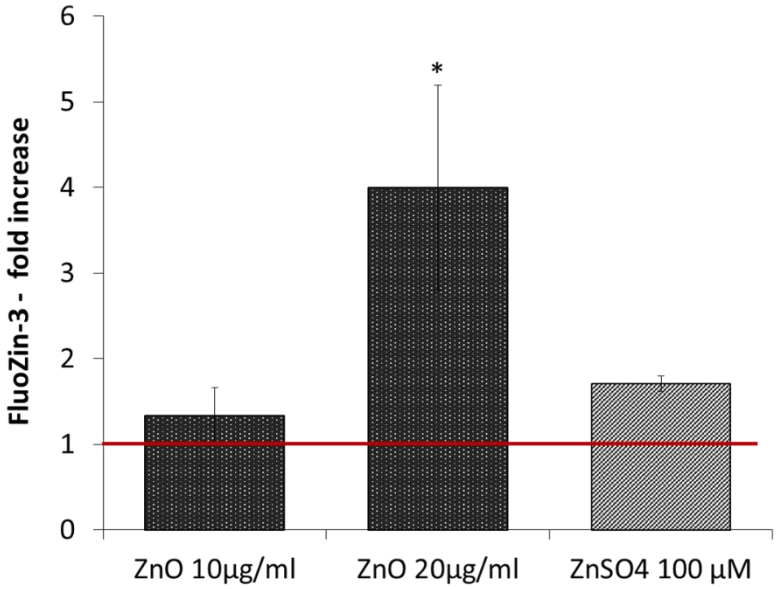


Fig. 4. Detection of free ions in NCI-H441 exposed to ZnO and $ZnSO_4$ salt. FluoZin-3 was loaded into the cells in order to measure intracellular free Zn^{2+}. The red line represents control value. Data are expressed as mean ± SE.*Statistically different between treated (nZnO and $ZnSO_4$) and control cells (ANOVA; $p < 0.05$).

3.5 Metallothionein (MT-I,II) expression

Since MT-I,II are one of the essential biomarkers in metal-induced toxicity, facilitating metal detoxification and protection from free radicals (Baird et al., 2006) the ability of nZnO to modulate the expression of MT was assessed. Data from western blot analysis showed that MT protein expression increases with nZnO doses, showing that the expression is significantly upregulated at higher concentrations (20 µg/ml) (Fig. 5 and Table 2).

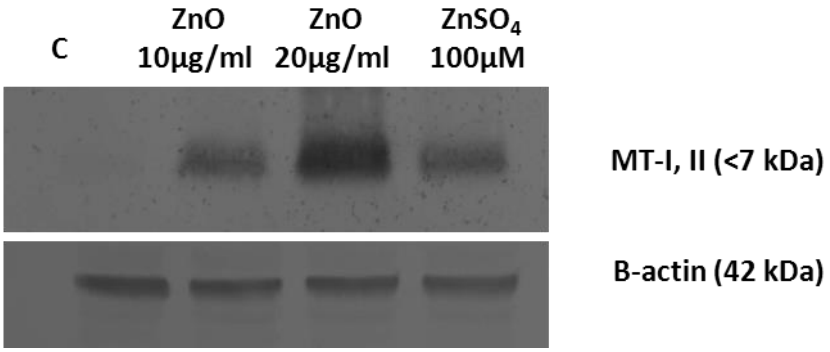


Fig. 5. Metallothionein (MT) protein expression. Representative western blot image of NCI-H441 cells exposed to 24h of nZnO.

Cell line	ZnO 10µg/ml	ZnO 20µg/ml	$ZnSO_4$ 100µM
NCI-H441	3.02±0.51	8.93±0.13	6.37±2.28

Table 2. MT-I,II protein expression. Representative values obtained by western blot analysis in NCI-H441 cells after 24h of nZnO exposure. Densitometric data were normalized to actin values and fold increases respect to the control are presented ± SD.

3.6 Cell morphology

Cells were stained with hematoxilin/eosin (HE) in order to evaluate nZnOs effects on the cellular morphology. Control cells are adjacent to each other and they form an epithelial monolayer (Fig. 6A and B). After treatment with 50 µg/ml nZnO for 6h, cells viability was not affected, as shown by the results on cell viability. However, cells show clear phenomena of cytoplasmatic vacuolization (Fig. 6C and D); furthermore rounded cells with condensate nuclei are visible, not present in the control cells.

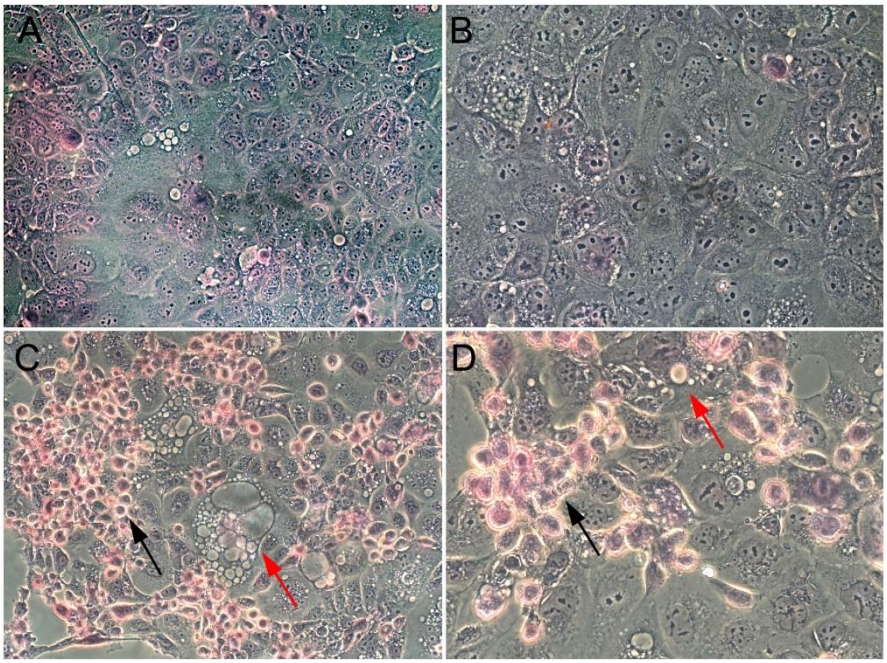


Fig. 6. Light microscopy of NCI-H441 monolayer. Cells were exposed to nZnO at 50µg/ml for 6h, stained with hematoxilin/eosin (HE) and observed with phase contrast microscope. (A) control cells; (C, D) nZnO treated cells showing a large number of rounded cells with condensed nuclei (black arrows) and vacuolization (red arrows). A, C= 20X magnification; B, D= 40X magnification.

3.7 Effects of nZnO on tight junction expression

Since previous studies have demonstrated the ability of nZnO to impair the integrity of the epithelial barrier *in vivo* (Bacchetta et al., 2014), we have evaluated the effects of nZnO on the expression of cellular junctions by the analysis of the TJ protein *zonula occludens-1* (ZO-1) ZO-1.

NCI-H441 have been treated for 6h with 50 µg/ml of nZnO and the expression and localization of the ZO-1 protein was determined by immunofluorescent staining. We used this concentration because previous studies have shown that nZnO affect the barrier integrity at high doses of exposure (Kim et al., 2010). Since in our previous tests cell viability at the maximum dose was seriously compromised (only 2% of viable cells) by the treatment for 24h, for this experiment cells were exposed to 50 µg/ml nZnO for 6h. The results obtained show that in control cells the expression of ZO-1 is located at the occludens junctions levels (Fig. 7A). Cells treated with nZnO had nuclei (in red) and confluence similar to the control, but the expression of ZO-1 resulted decreased and the localization of the protein at the cellular membranes discontinuous (Fig. 7B). These data demonstrate that nZnO are able to affect the junctional layout of epithelial cells.

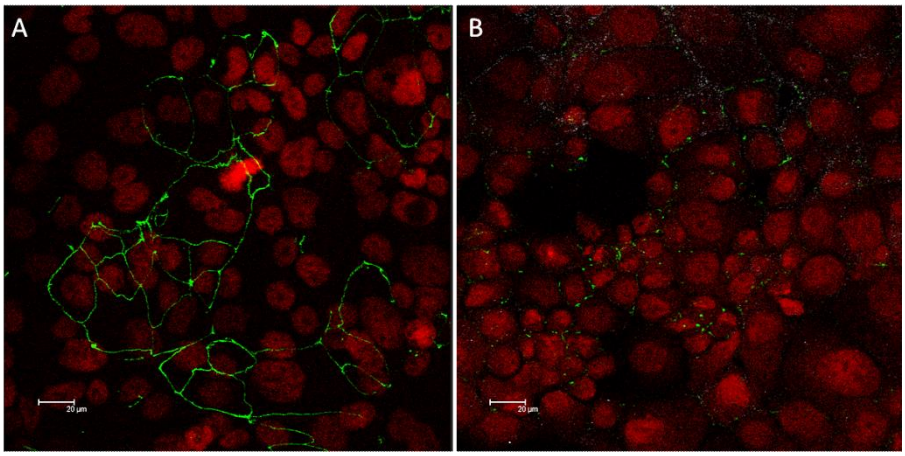


Fig. 7. ZO-1 expression. Immunofluorescent staining of the TJ cytoplasmatic plaque protein ZO-1 (green) of NCI-H441 cells monolayer control (A) and after exposure to 50µg/ml nZnO (B) for 6h. Nuclei were stained with Draq5 (red). NCI-H44 differentiated with 1 µM Dexamethasone after day 3 of culture were positively stained for ZO-1, after the treatment the expression is dramatically decreased. Scale bars= 20 µm.

3.8 Cellular uptake of nZnO

nZnO uptake in NCI-H441 cells was analyzed through indirect method by flow cytometry. Forward Scatter (FS) and Side Scatter (SS) parameters are related to cells morphological and physical characteristics. Furthermore, a change in cell morphology after the interaction with NPs can be an indicator of occurred uptake. FS is generally associated to cells dimensions, SS is strictly related to cell complexity, so it can be a sensitive indicator for the evaluation of NPs presence inside the cells. However, even particles located on cell surface can influence SS values. Dot plot in which the dispersion of cells is represented as function of FS (cellular dimensions) and SS (cells

granularity), after treatment with nZnO (50 µg/ml, 6h), showed a slight, but not significant, increase of SS (Fig. 8B).

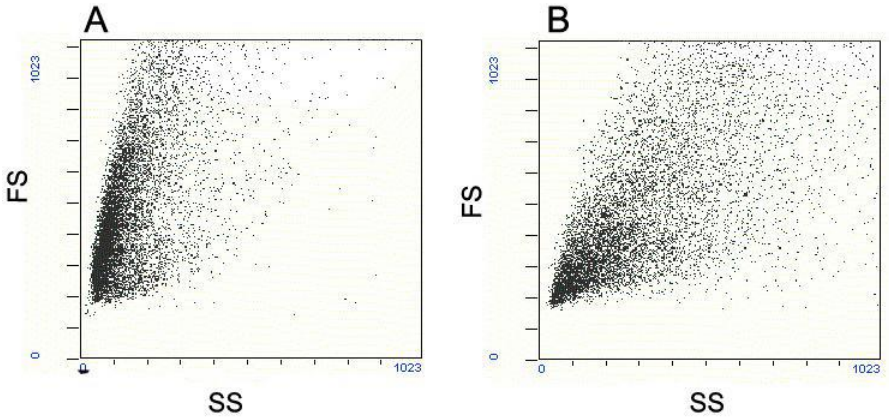


Fig 8. NPs uptake by flow cytometry. NCI-H441 cells exposed to nZnO at 50 µg/ml for 6h (B) were analyzed for light side scatter (SS) versus forward scatter (FS). Unexposed cells were scanned as controls (A).

nZnO uptake were also investigated through confocal microscopy (LeicaTCS NT). Cell scanning with laser reflection evidenced few nZnO (white spots) inside the cells and signals of particles localized also inside the nuclei of the cells (Fig. 9C, D). In control cells nanoparticles were not detected (Fig. 9A, B).

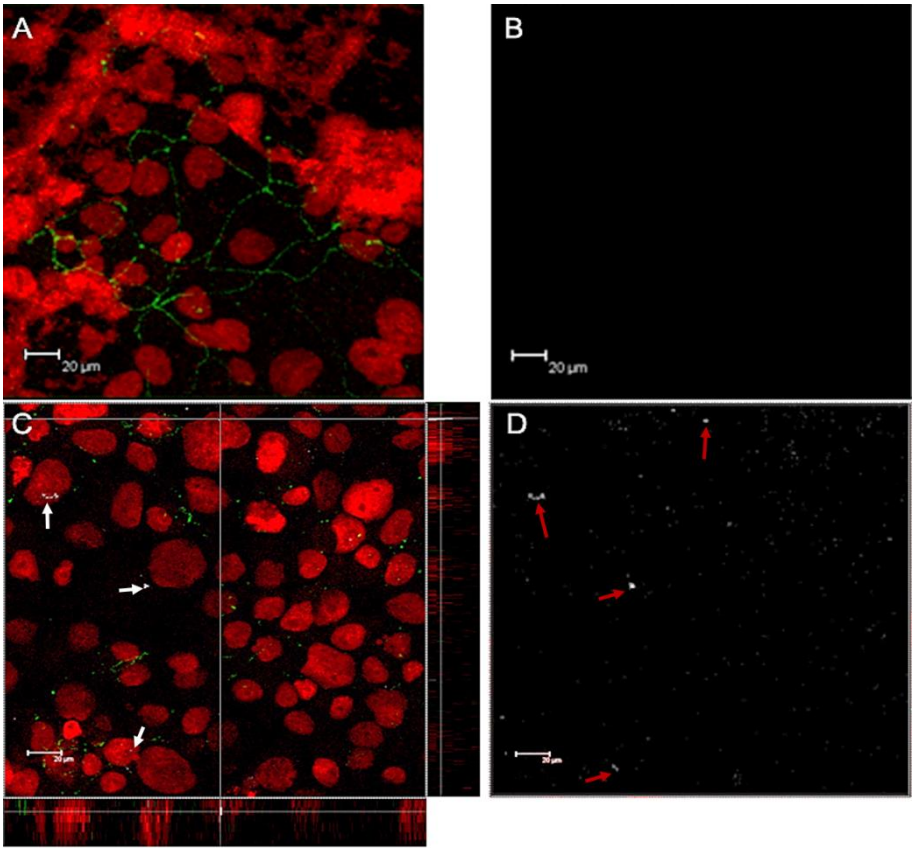


Fig 9. Confocal microscopy analysis of NCI-H441 cells. Control (A, B) and treated (C, D) cells with nZnO at 50 µg/ml for 6h were stained with Draq5 (red) and ZO-1 (green) and analyzed with confocal microscopy. White spots represent the internalized nZnO, as indicated by the white (C) and red arrows (D).

4. Discussion

nZnO, despite their wide application in many fields, have also cytotoxic effects, so potential health hazard should be taken into account. In the present study, nZnO (<50 nm, Sigma Aldrich, Italy) exposure significantly reduced cell viability of human lung cells NCI-H441, already at 20µg/ml

concentration. Cytotoxicity results are in accordance with previous studies, also in other lung cell lines (Sahu et al., 2013) and in cells coming from other tissues (Deng et al., 2009). Many *in vivo* and *in vitro* studies have reported that oxidative stress is a common mechanism for cell damage induced by NPs (Oberdörster et al., 2004; Nel et al., 2006; Ahamed et al., 2011). Similarly, in the present study, nZnO induced generation of ROS at the dose of 20µg/ml, when cells viability started to be significantly reduced, and that may be linked to the increased release of intracellular free Zn^{2+} induced by nZnO.

Data from detection of FluoZin-3, a sensitive indicator of intracellular free Zn^{2+} , showed that the exposure to nZnO (20 µg/ml) induced a significant increase of intracellular free ions while the exposure to $ZnSO_4*7H_2O$ (100 µM) induced a slight increase in Fluozin-3 intensity similar to the one detected after exposure to nZnO 10 µg/ml.

The complete dissolution of $ZnSO_4$ (100 µM) in culture medium determines a concentration of Zn^{2+} ions equal to 23 µM. This concentration is about 5 and 10 time lower than the maximum concentration of Zn^{2+} ions that could be released from nZnO 10 µg/ml (123 µM) or 20 µg/ml (246 µM) respectively.

Our results suggest that the increase in intracellular FluoZin-3 fluorescence after exposure to nZnO could be partially due to the uptake of extracellular Zn^{2+}. However, given the low cytotoxic effects of tested nZnO concentrations, we hypothesize that the intracellular increase in Fluozin-3 positive signals, after epithelial cells exposure to nZnO 20 µg/ml, might depend also on an intracellular dissolution of NPs and consequent release of Zn^{2+} ions. In accordance with the hypothesis of a "Trojan horse" (Limbach

et al., 2007) effect of nZnO we tested the cytotoxic effect of $ZnSO_4*7H_2O$ 200µM (data not shown). This salt concentration determined, after complete dissolution, a concentration of free Zn^{2+} equal to 46 µM and a cell mortality of 80%. Altogether these results suggest that dissolution of nZnO in cell culture medium is minimal and the extracellular free Zn^{2+} ions concentration is low enough to avoid extensive cell death.

Intracellular dissolution of metal based NPs and in particular nZnO is well documented in literature (Kim et al., 2010). Inside the cells internalized nZnO can be processed in lysosomal compartments (Cho et al., 2011) and dissolved with consequent release Zn^{2+}. The intracellular release of metal ions is also in accordance with MT over expression reported after the exposure to 20 µg/ml of nZnO. However it is not excluded that the induction of MT may be due also to the presence inside the cells of nZnO themselves.

We also observed that MT activation induced by the salt is higher compared to 10 µg/ml of nZnO, in accordance to the major availability of intracellular Zn^{2+} released from the salt, as suggested by Fluozin-3 results. However, further experiments will be required in order to evaluate the amount of Zn^{2+} released by nZnO and to better localize NPs in the cellular compartment, focusing primarily on lysosomes.

Our results about the potential of nZnO to induce MT expression are in accordance with previous *in vivo* and *in vitro* works. *In vivo* studies have shown that exposure of rats to 5 and 2.5 mg/m^3 of nZnO resulted in increased MT mRNA levels (compared to air-exposed control animal values) immediately after exposure, as early response to nZnO (Cosma et al., 1992; Beyerle et al., 2010). However in our study we reported the

expression of MT at the protein level, that may occur at later times respect to gene expression. Furthermore, also Sahu et al. (2013) have demonstrated that nZnO exposed cells for 24h showed up-regulation of the MT gene.

Study on cells morphology and junctional protein ZO-1 expression have been further assessed. Bacchetta et al. (2014) has also shown that nZnO are able to alter the ZO-1 expression in *Xenopus leavis* gut, resulting in a decreased permeability of the gastro-intestinal barrier. *In vitro* studies have demonstrated that nZnO are able to alter the cell morphology by affecting the junctional complex (Kim et al., 2010). In accordance with these results, our studies have evidenced that after 6h of nZnO exposure (50µg/ml) cells are rounded and shrank, even if they maintained confluence, and with evident vacuolization., indicating that nZnO induce morphological changes that can compromise the epithelial barrier integrity. Furthermore, also ZO-1 protein expression at the membranes decreased. These data confirm that nZnO induces several biological responses that have negative effects on the cells, even when cells viability is not seriously compromised by the treatments.

5. Conclusions

Although additional studies are required to further delineate specific mechanisms underlying cytotoxicity of nZnO and to understand the role of release Zn^{2+} in mediated toxicity, in the present study we showed the strict relation among nZnO induced cytotoxicity, intracellular ions release and ROS formation, also showing the ability of cells to activate mechanisms of protection (MT). Furthermore nZnO were able to change the morphology of the cells, with evident alteration also at their ability to form tight junctions.

According to these data and previous works, nZnO are harmful for lung cells and they pose a hazard for humans that are unintentionally exposed to such kind of particles, especially for workers that daily deal with them. Furthermore, since zinc typically appears in the form of ZnO in ambient particulate matter (Adamson et al., 2000; Councell et al; 2004) and the emission in the environment of nZnO-based NMs is highly increased in the last decade (Piccinno et al., 2012), a better knowledge about NPs-induced mechanism is becoming pivotal in order to design safer NPs for the humans and the environment.

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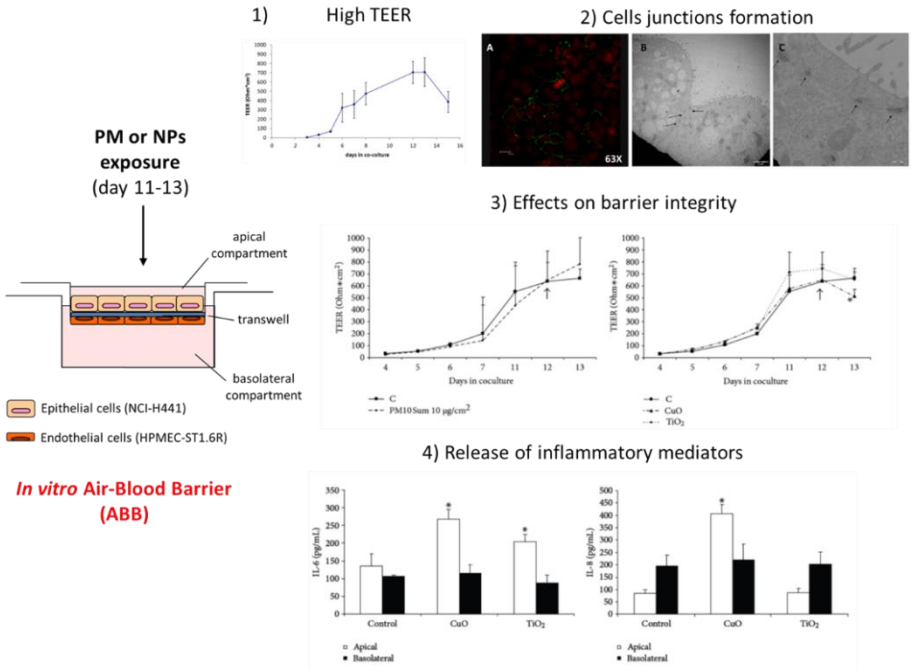
CHAPTER IV

Air-blood barrier: *in vitro* set-up to assess particles toxicity

Graphical abstract

In this chapter a graphical abstract of the paper published about the complete set-up of the model and the effects of PM and different NPs is presented.



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Research Article

Effect of Nanoparticles and Environmental Particles on a Cocultures Model of the Air-Blood Barrier

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Abstract

Exposure to engineered nanoparticles (NPs) and to ambient particles (PM) has increased significantly. During the last decades the application of nano-objects to daily-life goods and the emissions produced in highly urbanized cities have considerably augmented. As a consequence, the understanding of

the possible effects of NPs and PM on human respiratory system and particularly on the air-blood barrier (ABB) has become of primary interest. The crosstalk between lung epithelial cells and underlying endothelial cells is indeed essential in determining the effects of inhaled particles. Here we report the effects of metal oxides NPs (CuO and TiO_2) and of PM on an *in vitro* model of the ABB constituted by the type II epithelial cell line (NCI-H441) and the endothelial one (HPMECST1.6R). The results demonstrate that apical exposure of alveolar cells induces significant modulation of proinflammatory proteins also in endothelial cells.

1. Introduction

Diffusion biological barriers, such as epidermis, the gastrointestinal tract, and the respiratory epithelium, are physiologically designed to separate two different compartments in order to allow the selective passage of external substances that are essential for the organisms and to protect the body from pathogens and hazardous substances. The alveolar region is the functional area for the gas exchange. Alveolar epithelial cells form tight and adherens junctions which play a key role in the functionality of the air-blood barrier (ABB). In addition the maintenance of the integrity of the ABB is pivotal for the physiological function of the lungs and limits the passage of inhaled material to the blood circulation. The alveolar surface is approximately 100 m^2 and thus is the primary target for inhaled particles and nanoparticles. Inhaled particulate interact with the apical side of the epithelial cells and might be internalised and eventually translocated to the endothelium and to the blood circulation. Moreover, even in the absence of translocation, cell-particle interactions might determine an inflammatory status that in turn

facilitates particles translocation due to the loss of the selective permeability of the epithelial cells and to an increase of the permeability of the endothelial cells [1, 2]. Several *in vivo* studies have reported pulmonary inflammation and lung injury in response to nanoparticles (NP) exposure. Nevertheless the specific effects on the ABB are scanty due to the complexity of a precise anatomical analysis of this region of the lung. As an alternative, simpler *in vitro* models of alveolar epithelium have been widely used to study important biological and functional characteristics. However the specificity of the ABB deserves more accurate analyses possibly developing a model able to mimic the interplay of epithelial and endothelial cells. NPs have gained major attention in the last years due to the increase of their application in commercial products and biomedicine and the increased evidence that metal oxide particles are present in the environment as a consequence of human activities. Furthermore metal NPs have found in the last years an extensive use in industrial applications and as a consequence concern has been raised on their potential adverse effects on humans after accidental inhalation. Moreover monitoring analyses have highlighted the high presence of metal oxide NPs at sites surrounding factories, when compared to clean areas [3], and epidemiological studies have reported a correlation between the level of such NPs and the increase in pulmonary disease including exacerbation of bronchial asthma [4]. *In vitro* studies have reported cytotoxic effects for CuO NPs [5] while TiO2 NPs have been usually referred to as particles without significant biological effects [6]. However the results may be different depending on many factors such as the cell types used, NPs administration conditions, and the test sensitivity. Furthermore NPs may induce alveolar-capillary barrier injury causing

disruption of alveolar and endothelial integrity which may determine the leakage of inflammatory mediators in the circulatory system [7, 8]. Particulate matter (PM) is considered one of the major environmental contaminants in different cities, and although great attention has been devoted to the fine fractions of PM, a main issue remains the potential effects of the coarse fraction (PM10) which has been reported to possess specific chemical and biological properties [9, 10]. In fact summer PM10 samples have been reported to be characterized by bacterial components and crustal elements, responsible for the production of proinflammatory effects. In the present paper the effects of two metal oxide NPs (CuO and TiO_2) and of summer PM10 sampled in Milan on a model of ABB are presented. The model consists of a human lung epithelial cell line, NCI-H441, with characteristics of both type II pneumocytes and Clara cells [11] and the human pulmonary microvascular endothelial cell line (HPMECST1.6R) cultured on opposite site of a transwell filter insert [12, 13]. The ABB model has been characterized for its functional properties while particles effects have been determined by measuring the transepithelial resistance (TEER) and the release of pro-inflammatory mediators. Internalization of nanoparticles has been furthermore assessed by means of transmission electron microscopy.

2. Materials and Methods

2.1. Preparation of NPs Suspensions.

nCuO (<50 nm) and $nTiO_2$ (<100 nm) (Sigma Aldrich, Italy) were weighted and suspended in ultrapure sterile Milli-Q water at the concentration of 8 mg/mL. Suspensions were sonicated for 1 min with the sonicator bath

Ultrasonic Soniprep 150 MSE (Sanyo) and then diluted in PBS + BSA (0,1% final concentration; Sigma Aldrich) in order to avoid particles agglomeration and optimize suspension stability. Working concentrations (0, 1, 10, 25, 50, 100 μg/mL) were obtained by adding NP suspensions directly to the culture medium (OptiMEM1X, 1% FBS; M199, 1% FBS; GIBCO). NPs and NPs suspensions were characterized as reported [14]. PM10 collected and characterized as previously reported [10] has been resuspended in sterile water and added directly to the culture medium.

2.2. Cells Cultures.

Human pulmonary microvascular endothelial cell line HPMEC-ST1.6R was received from Dr. Ronald E. Unger (Institute of Pathology, Medical University of Mainz, Johannes Gutenberg University, Mainz, Germany) and cultured in 0,2% gelatine (Sigma Aldrich) coated flasks in M199 medium supplemented with 15% FBS (Gibco), 2 mM Glutamax I (Sigma Aldrich), 25 μg/mL sodium heparin (Sigma Aldrich; Italy), 25 μg/mL endothelial cell growth supplements (Sigma Aldrich), and 100 U/100 μg/mL Pen/Strep (Euroclone, Italy) at 37∘C, 5% CO_2. The human lung adenocarcinoma cell line NCI-H441 (ATCC, USA) was maintained in OptiMEM medium (Gibco) supplemented with 10% FBS and Pen/Strep (100 U/100 μg/mL), at 37°C, 5% CO_2.

2.3. In Vitro Alveolar-Capillary Barrier Model: Coculture of HPMEC-ST1.6R with NCI-H441.

HPMEC-ST1.6R ($9\times10^4/cm^2$) cells were placed on the lower surface of Transwell filter membranes (polyester; 0,4 μm pore size; Costar) coated

with 0.2% gelatine and incubated for 2 h at 37°C and 5% CO_2. The filter membranes were then turned upside down and placed in a 12-well plate filled with 1,5 mL HPMEC-ST1.6R medium (15% FBS) and incubated for 24 h. The day after NCI-H441 ($2 \times 10^4/cm^2$) cells were seeded on the top surface of the transwell filters and cultured to confluence simultaneously with the HPMEC-ST1.6R seeded on the lower surface for 11 to 13 days. To induce differentiation, the NCI-H441 cell line in cocultures was treated with 1 μM Dexamethasone (Sigma Aldrich) from day 3 of cultivation. Summer PM10 (10 $\mu g/cm^2$ equivalent to 22,5 μg/mL) and nCuO and nTiO2 suspensions (25 μg/mL) were added to the apical compartment at the 11th/12th day of coculture for additional 24 h. the doses of treatment have been defined accordingly to previous results we have reported for PM10 effects [10] and by dose-response curves on the single cell lines used for setting up the air-blood barrier model (data not shown).

2.4. Transepithelial Electrical Resistance (TEER) Measurements.

In order to determine the integrity of the *in vitro* barrier the transbilayer electrical resistance (TEER) was measured with an EVOM Volt Ohm Meter (World Precision Instruments, Berlin, Germany) equipped with a EndOhm Chamber (World Precision Instruments, Berlin, Germany). After PM and NPs exposure, before TEER measurements, transwell filters were washed with PBS and incubated with M199 medium for 10 min in order to avoid alteration in TEER values due to cellular debris and to insert handling. The TEER of polyester transwell filter membranes coated with gelatine 0.2%, without cells, was measured and set as blank. Barrier resistance readings ($\Omega * cm^2$) were made by subtracting the resistance of the blank filter

membrane and by multiplying the area of the insert (1.12 cm^2). Resistance was reported as mean ± SE of at least three independent experiments.

2.5. Immunostaining.

After 11-12 days of cultivation the apical compartment of coculture was washed in PBS and then fixed with paraformaldehyde (1%) for 20 min at RT. Cells were then permeabilized with Triton X-100 1%, TWEEN 1% in PBS + BSA 3% for 20 min at 4∘C. the cells were then washed in PBS and incubated overnight at 4∘C with rabbit anti-human *zonula occludens-1* (ZO-1) antibody (cell signaling; 1 : 100) in PBS + BSA 1% buffer. Cells were then washed three times in PBS and then incubated with the secondary antibody goat anti-rabbit Alexa fluor-488 (Invitrogen Molecular Probes Srl; 1 : 1000) for 1 h. For actin staining cells were incubated 1 h with phalloidin-TRIC (Sigma Aldrich, 1 : 750). Samples were mounted on a glass slide with ProLong mount (Invitrogen Srl) and observed by an AxioScope reverted microscope (Zeiss, Germany).

2.6. Cytokines Release.

At the end of the exposure time cell culture media were collected and centrifuged at 1100 rpm for 5 min to remove cell debris. The final supernatants were stored at −80°C. IL-1β, IL-6, and IL-8 protein levels were determined by sandwich ELISA (Human Cytoset IL-1β, IL-6 and IL-8; Biolegend) according to the manufacturer’ s instructions. Absorbance was measured and quantified by a plate reader (Multiskan Ascent, Thermo Scientific Instruments) at wavelengths of 450 nm and 570 nm.

2.7. Nanoparticles Uptake.

Coculture samples were prepared for transmission electron microscopy (TEM) according to the standard procedures. Briefly, cells were fixed in 2,5% glutaraldehyde for 20 min at 4°C and postfixed with 1% osmium tetroxide for 1 h, followed by dehydration in graded ethanols. Samples were then embedded in Epon/Araldite resin, and ultrathin sections were cut at the ultramicrotome (Reichert Ultracut Jung E). the sections were collected on copper grids, counterstained by lead citrate and uranyl acetate, and exanimated by Jeol JEM 1220 TEM microscope operating at 80 kV equipped with a Gatan CCD camera for digital imaging.

2.8. Statistical Analysis.

All the experiments have been performed in independent triplicates, and data are reported as mean ± S.E. if not otherwise specified. Statistical analyses have been performed with Sigmastat 3.1 software with ANOVA test and post hoc analysis.

3. Results

3.1. Formation of the Alveolar-Capillary Barrier.

In order to estimate the integrity of the *in vitro* alveolar-capillary barrier the formation of tight junctions (TJs) was observed and the transepithelial electric resistance between cocultures measured. The TJs formation was assessed by the stain of the tight junctional cytoplasmic plaque protein ZO-1. Data showed that after 11‑13 days of cocultures and stimulation with Dexamethazone (1 μM), ZO-1 junctions were localized at the periphery of NCI-H441 cells as a continuous line delineating the limits of each cell

(Figure 1(a)). The importance of the Dexamethazone stimulation is evident when comparing the ZO-1 staining of nonstimulated monolayer in which it is impossible to identify a clear membrane localization of ZO-1. The formation of junctions between adjacent alveolar cells (NCI-H441) was also evident by transmission electron microscope analysis (Figure 2).

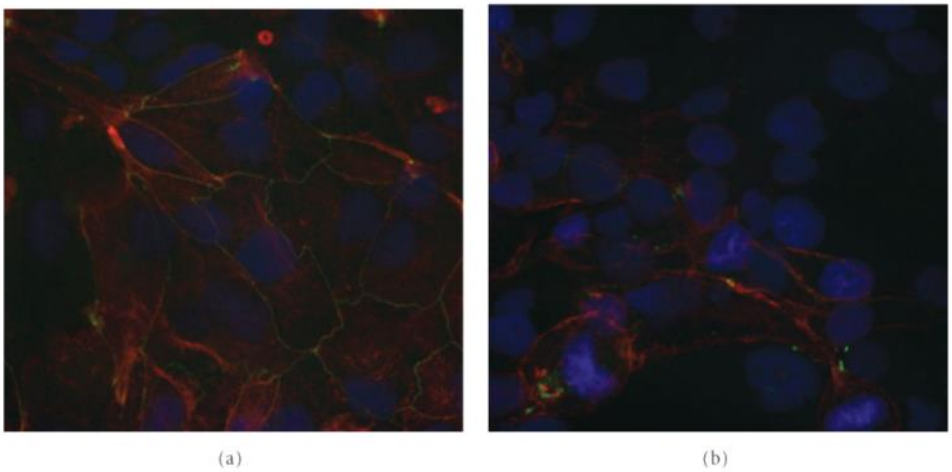

FIGURE 1: Immunofluorescent staining of the TJ cytoplasmic plaque protein ZO-1 (green) of the NCI-H441 monolayer on day 12, performed as described in Section 2. NCI-h441 differentiated with 1 μM Dexamethasone after day 3 of culture were positively stained for ZO-1 at the cell-cell interface (a) confirming the formation of functional TJs while in cells without Dexamethasone treatment the ZO-1 staining clearly demonstrates the absence of TJs formation; (b) nuclei were stained with DAPI (blue), ZO-1 (green), and actin (red).

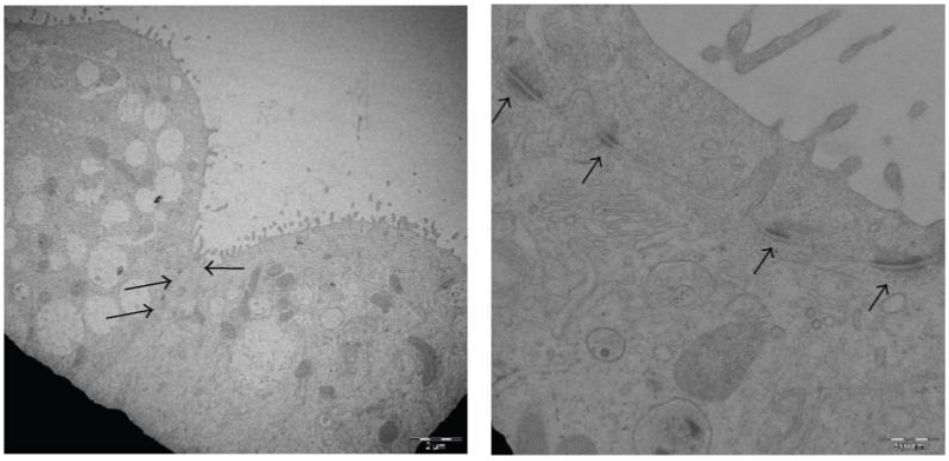

FIGURE 2: (a) and (b) Transmission electron microscope of NCI-H441 cells after 12 days of coculture. The TEM pictures show the formation of tight (↑) and adherens (⇑) junctions.

The integrity of the *in vitro* alveolar barrier was assessed by measuring the transepithelial electric resistance (TEER) between the cocultures. TEER was expressed as Ohm∗cm^2, and the data showed that NCI-H441 and HPMEC-ST1.6R cocultures reached the maximum TEER at 703 ± 118 Ω∗cm^2 (N=7) after 11‑13 days of cultures (Figure 3). Preliminary data showed that the treatments with PM10 (10 µg/cm^2 equivalent to 22.5 µg/mL) and with $nTiO_2$ (25 µg/mL) did not produce significant changes in the TEER values (Figures 4(a) and 4(b)). On the contrary the TEER of the ABB treated with nCuO showed a significant reduction. The TEER value after 24 h of treatment was reduced to 514 Ω∗cm^2 compared to the control value of 665 Ω∗cm^2 (Figure 4(b)).

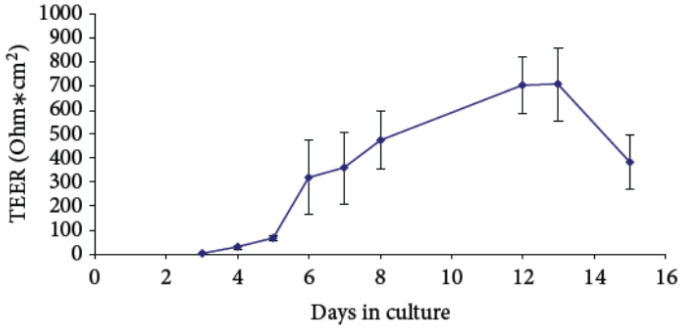

FIGURE 3: Transepithelial electric resistance (TEER) values of the ABB coculture of NCI-H441 (treated at day 3 with Dexamethasone 1 μM) and HPMEC-ST1.6R. TEER values are expressed as $\Omega*cm^2$. The highest values of TEER were reached after 11–13 days in culture (703 ± 118 $\Omega*cm^2$). Gelatine-coated inserts without cells were used as blank. Data are expressed as means ± S.E. of seven different experiments.

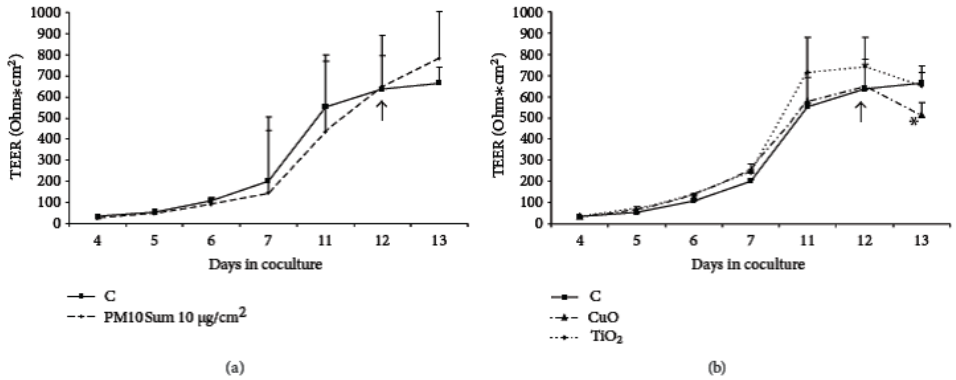

FIGURE 4: Transepithelial electric resistance (TEER) values of the ABB coculture of NCI-H441 (treated at day 3 with Dexamethasone 1 μM) and HPMEC-ST1.6R. The ABB has been apically treated with PM10 (Figure 4(a)) and metal oxide NPs (Figure 4(b)). TEER values are expressed as $\Omega*cm^2$ and show no significant differences between control and PM10 and TiO_2-treated cells. Cells treated with CuO NPs showed a significant reduction in TEER values. ↑ indicates the day of apical treatment with particles. Gelatine-coated inserts without cells were used as blanks. Data are expressed as means ± SE of 3 different experiments. ∗Statistically different from control $P<0.05$, ANOVA.

3.2. Cocultures Exposure to nCuO, nTiO2, and Summer PM10: Iflammatory Response.

The ABB coculture system was treated with summer PM10 and metal oxide NPs (nCuO and $nTiO_2$) in order to evaluate the specific inflammatory effects produced to the ABB model. The results showed that summerPM10 induced a significant increase in IL-1β release from basolateral compartment (158 pg/mL versus 28 pg/mL of the control group) while the apical compartment did not produce any IL-1β release (Figure 5).

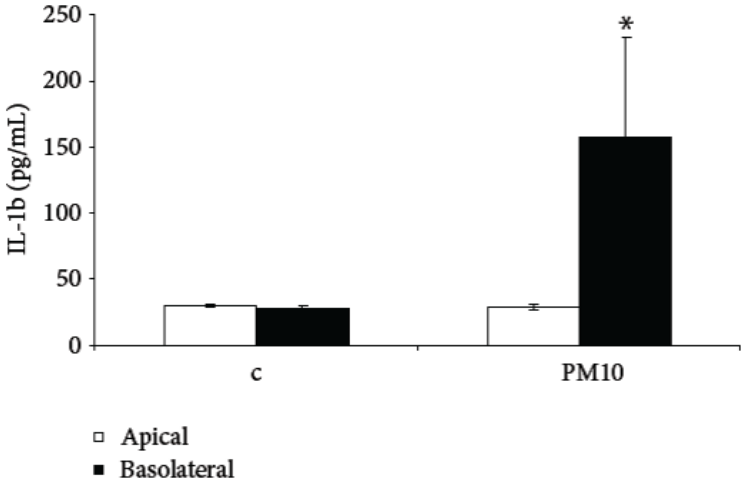

FIGURE 5: IL-1β release from the ABB model exposed to Milan summer PM10 for 24 h at day 12 of culture. The release of the interleukin is significantly increased in the basolateral compartment (endothelial cells) after apical exposure of the system (NCI-H441 compartment). $*$Statistically different from control $P<0.05$, ANOVA

The ABB treated with CuO showed a significant increase in IL-6 and in IL-8 release (268 pg/mL of IL-6 in treated cells supernatants versus 136 pg/m in control ones and 406 pg/mL of IL-8 in treated cells supernatants versus 83 pg/m in control ones) from the apical compartment while a significant interleukins release from the basolateral compartment was not observed (Figure 6(a)). $nTiO_2$ induced an increase only in IL-6 (205 pg/mL in the treated group versus 136 pg/mL in control one) from the epithelial compartment (Figure 6(b)). NPs failed to induce significant release of IL-1β (data not shown).

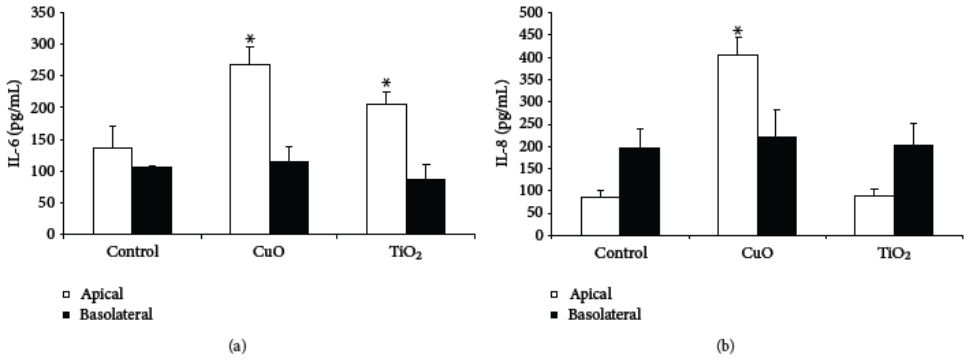

FIGURE 6: IL-6 and IL-8 release from the ABB model exposfied to metal oxides NPs (25 μg/mL) at day 12 of culture. The release of IL-6 is signicantly increased by CuO and TiO_2 NPs in the apical compartment (a) while IL-8 is significantly increased in the apical compartment only after treatment with CuO NPs. (b) ∗Statistically different from control $P<0.05$, ANOVA.

3.3. NPs Internalization in Cocultures.

NCI-H441 cells of coculture experiments exposed to CuO and TiO_2 NPs showed the internalization of CuO and TiO2 NPs. Interestingly nCuO was found free in the cytoplasm (Figure 7(a)) while $nTiO_2$ (Figure 7(b)) was compartmentalised into vesicles.

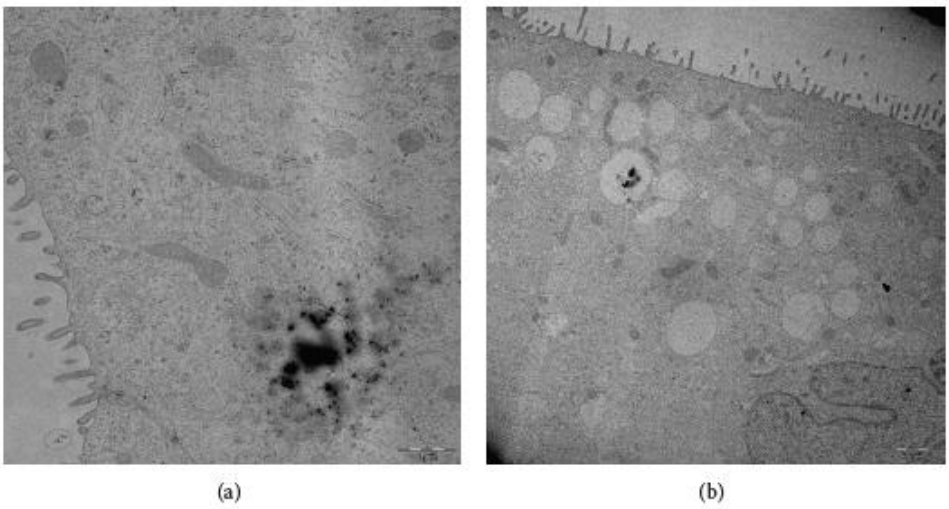

FIGURE 7: Transmission electron microscope of NCI-H441 cells after 12 days of cocultures treated with NPs. (a) NCI-H441 cells treated with CuO (25 μg/mL). (b) NCI-H441 cells treated with TiO2 (25 μg/mL). The presence of CuO NPs free in the cytoplasm can be related to the oxidative potential of these particles which determines the rupture of endosomal vesicles as well as to a different route of entry into the cells.

4. Discussion

4.1. Coculture Model of In Vitro Air-Blood Barrier.

The need of developing new models to investigate the effect mechanisms produced by inhaled toxicants has been also recently outlined [15]. The gold standard proposed for the ABB is a coculture of primary alveolar epithelial cells and human pulmonary endothelial cells [16]. Such model is expensive and subject to differences due to the primary cells availability. Therefore the development of an ABB *in vitro* system replicating the architecture and functionality of the alveolar space, based on cell lines, is of particular interest for the reduction of *in vivo* experiment too. Here we present the data obtained by the use of a model constituted of two cell types: the alveolar type II cell line, NCI-H441 and the human pulmonary microvascular endothelial immortalised cells, HPMEC-ST1.6R. NCI-H411 is a commercially available line while HPMEC-ST1.6R has been obtained by primary human pulmonary microcirculation endothelial cells (HPMEC) transfected with the plasmids pSV3neo and pC1.neo.hTERT [17]. These endothelial cells exhibited most of the characteristics of the primary human pulmonary microvascular endothelial cultured cells [13] and have been therefore selected as representative model to establish a functional ABB. The coculture model reached, after 11‑13 days, a TEER representative of a functional barrier with a maximum of 703 ± 118 $\Omega*cm^2$, in agreement with previous reports [8, 12]. Such TEER value is reached thanks to the tight junctions (TJs) and adherens junctions (AJs) usually formed between epithelial cells via highly regulated events that establish cell differentiation (apical-basolateral membrane polarity) [18]. The immunofluorescence technique, used to evidence the epithelial cells Zonula occludens-1 (ZO-1),

confirmed the integrity of the ABB. ZO-1 is one of the major cytoplasmic proteins forming the TJs and has been found to be associated with the transmembrane protein occludin and to link it to the actin-based cytoskeleton [19]. After 11‑13 days of cocultures and stimulation with Dexamethazone (1 μM), ZO-1 junctions were clearly localized at the periphery of NCI-H441 cells as a continuous line surrounding each cell at its border. Moreover the transmission electron microscope images confirmed the formation of AJs among adjacent cells. The TEER of the ABB models, exposed to TiO_2 NPs or to PM10, showed no differences with respect to the controls. These data confirm that the low doses used did not induce significant cell toxicity in the epithelial cell line. On the contrary CuO NPs induced significant effects on TEER, therefore confirming the ability of the NPs to affect the functionality of the AAB. Indeed in A549 cells the same dose of nanometric CuO have been reported to induce a 30% of cell viability reduction [14, 20]. In other cell lines, CHO and HeLa cells, CuO NPs resulted to be more toxic, and the toxicity was related to the dissolution of Cu^{2+} ions from the particles [21]. The differences, among the effects produced on the cell lines used, are likely due to different uptake processes and/or different responsiveness to the treatment. Recently it has been reported [8] that coculture systems are less sensitive to NPs-induced cytotoxicity, therefore, the low reduction of TEER here observed could be related to an overall lower responsiveness of the NCI-441 cell line when differentiated for the ABB model. Nevertheless the ability of CuO NPs to induce a TEER reduction underlines the importance of better understanding the effects of NPs on the ABB in terms of reduction of its functionality.

4.2 Inflammatory response.

Inflammation processes are general responses to different lung diseases, even inflammation is a physiological response, and an uncontrolled inflammatory status may lead to adverse health effects. The ability of inhaled particles to induce the release of proinflammatory mediators has been largely used as marker of potential toxic effects [22, 23]. However the data available refer to monoculture of lung epithelial cells and the few data exist on lung endothelial cells. the biochemical crosstalks between the cell types constituting the ABB are fundamental in maintaining the barrier homeostasis [24‑26]. The NPs ability to induce an acute inflammatory response in lungs of rodents has been reported [27, 28]. *In vitro* monocultures of both lung epithelial and endothelial cells have been used to define the proinflammatory potential of metal oxide NPs [23, 29‑31]. Nevertheless the data on significant model of ABB are rather scanty [8]. In agreement with our previous results [14] the ability of CuO and TiO_2 NPs to induce a significant inflammatory response in the apical side of the ABB mode is shown. CuO NPs induced a significant release of the proinflammatory mediators, IL-6 and IL-8, and TiO2 increased only the release of IL-6 while both the NPs did not induce significant release of IL-1β. The lack of response of the basolateral side can be related to the integrity of the ABB which prevents the passage of NPs and/or ions towards the endothelial compartment although additional data are needed to elucidate the basolateral responses. However, Papritz et al. [32] showed for Cd^{2+} ions that, after apical exposure, the basolateral response was significant at concentrations which determined a decrease in TEER values. Interestingly the apical treatment of the ABB with PM10 induced a

basolateral release of IL-1β. This result is in accordance with others [8, 16] which obtained a basolateral response of endothelial cells after treatment of the epithelial cells at the apical side. IL-1β release confirms the biochemical crosstalk between the two sides of the membrane and supports the functionality and representativeness of our ABB model. Further studies will be needed to understand the mechanism by which PM10 induces IL-1β. Preliminary results obtained in our lab on THP-1-derived macrophages suggest that the inflammasome activation plays a central role (data not shown). the ability of PM in triggering the release of a potent inflammatory mediator such as IL-1β has to be carefully taken into account. Van Eeden et al. [33] showed a significant increase of circulating IL-1β in population exposed to high level of PM10; furthermore increased expression of IL-1β has been related to lung injury and lung tissue remodelling [34]. Our ABB model thus furnishes a suitable system to be used for studying lung damage mechanisms and systemic inflammation following exposure to environmental and engineered NPs.

4.3. NPs Internalization in Cocultures.

Several *in vivo* and *in vitro* studies showed the translocation of NPs through the respiratory epithelium and the distribution of NPs in secondary organs such as liver, kidney, and brain [35]. Nevertheless there are still controversial evidences about NPs translocation through the alveolar barrier. ABB *in vitro* models can thus furnish a helpful system for investigating the actual translocation process across the barrier. Intake of submicrometric and nanometric particles in culture of epithelial cell has been widely reported [36‑38]. Our data showed that both nCuO and nTiO2 were found

internalised in epithelial cells; however while nTiO2 particles were found as agglomerates in cytosolic vesicles, suggesting an endocytosis mediated mechanism of internalization, nCuO particles were found as free aggregates in the cytoplasm [14, 39]. nCuO were found as free aggregates into the cytoplasm. this difference might be related to the higher oxidative potential of CuO NPs in comparison with TiO2 ones, and this ability may lead to a lipid peroxidation in the endosome vesicles, with a consequent presence of free CuO NPs in the cytoplasm. However after 24 h of treatment, NPs were not found internalised in the basolateral compartment of endothelial cells. The inability of nCuO and nTiO2 to cross the membrane filter pores of 0.4 μm can be due to the tendency of NPs to form aggregates that are physically unable to pass through the membrane pores. Indeed it has been proposed that, even without a cell layer on the top of the filter, NPs which tend to agglomerate are not able to cross the membrane [40]. The same authors have demonstrated the capability of certain NPs to cross an *in vitro* model of ABB, in experiments that used a membrane filter with 3 μm pore size and A549 cell line, which were not able to form TJs. Our data thus suggest that in presence of a functional ABB, the possibility of NPs to cross the barrier is rather scanty, although additional data are needed. For example, the use of air-liquid interface treatments, which drastically reduce the NPs agglomeration tendency in culture media, will be of particular interest in defining the translocation ability of nano objects.

In conclusion the ABB model reported here is a suitable system for studying the toxicological events at the alveolar level. Our date demonstrate that the two cell types are able to cross communicate, as evidenced by the IL-1β release in PM10-treated samples, although the passage of particles seems

not to occur. Further studies will help to understand which molecules are responsible for the cells crosstalk and if, under different cell treatment conditions, the NPs translocation is really possible in a functional ABB.

Acknowledgment

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CHAPTER V

Impact of nZnO on *in vitro* models of the human air-blood barrier

Abstract

The inhalation of zinc oxide NPs (nZnO) induces systemic disease (i.e., metal fume fever), damages to the alveolar epithelium and inflammatory response in endothelial cells. Even though some of these findings have been confirmed by *in vivo* and *in vitro* experiments, *in vitro* models of the air-blood barrier (ABB) are needed to elucidate the biological mechanisms underlying the effects of inhaled NPs. An ABB *in vitro* model, consisting of a Transwell co-culture of lung epithelial cell lines (NCI-H441) and immortalized pulmonary microvascular endothelial cells (HPMEC-ST1.6R), was set up. In addition, a tri-culture model was developed by adding monocytes (THP-1) on the basal compartment of the inserts. These models have been set up to analyse the interplay among different cell types in determining biological outcomes after exposure to nZnO, such as inflammation, endothelial damage and modulation by cells from the immune system. The barrier integrity was assessed by measuring the transepithelial resistance (TEER); ELISA test was used to analyse the pro-inflammatory response of both apical and basolateral compartments of the models. Data obtained demonstrated that nZnO exposure did not affect the barrier integrity (no reduction of TEER after 24h), but induced an activation of endothelial cells with the release of pro-inflammatory mediators (IL-6, IL-8) and endothelial dysfunction markers (sICAM-1 and sVCAM-1). These results confirm that apical exposure to NPs may determine endothelium activation. The *in vitro*-ABB model presented is a useful tool

for understanding the lung epithelium and endothelium interaction in NPs-induced biological response, and to determine the importance of endothelium dysfunction in response to NPs inhalation**.**

1. Introduction

Inhalation is the major route for environmental airborne toxic insults. Exposure to particulate matter (PM) and NPs (NPs) by inhalation has been related to respiratory, cardiovascular and systemic effects (Li et al., 2008; LeBlanc et al., 2009; Nurkiewicz et al., 2011, Nemmar et al., 2002).

The mechanisms behind these effects are still unclear, but several *in vitro* studies have provided evidence of the specific cellular effects that are linked to the *in vivo* outcomes (Blank et al., 2007; Alfaro-Moreno et al., 2008; Herseth et al., 2008).

In vitro cell cultures of the respiratory system have been frequently used. Cell lines such as Calu-3, which consist of bronchial cells (Geys et al., 2006), or A549, which are type II pneumocyte-like cells (Alfaro-Moreno et al., 2002), were largely used to investigate biological responses after exposure to particles. Other studies have used cells from different tissue targets, such as endothelial cells HUVEC (Montiel-Dávalos et al., 2012), cardiac cells (Totlandsdal et al., 2008), and neurons (Wu et al., 2010). The main concern regarding using cells not belonging to the respiratory tract is that they do not represent a situation that closely mimics the *in vivo* one. Furthermore, in monoculture systems there would not be quantification of the amount of particles that may translocate from the respiratory tract to the blood stream and to other organs (Kreyling et al., 2002; Nemmar et al., 2001; Geiser et al., 2005). Despite this limitation, available *in vitro* studies

have been very useful in understanding the possible mechanisms behind the systemic effects of PM and NMs (Geiser et al., 2005; Alfaro-Moreno et al., 2008; Napierska et al., 2012).

The respiratory barrier has an important role in the study of pulmonary and cardiovascular toxicity. In order to mimic the lung-blood barrier, several components from the pulmonary compartment and pulmonary circulation can be incorporated in an *in vitro* model. The alveolar compartment mainly consists of alveolar type I cells (AEICs) and type II epithelial cells (AEIICs). AEICs are squamous thin differentiated cells, and they cover the 90-95% of the alveolar cells. AEIICs are cuboidal cells that constitute the 15% of lung cells and are considered progenitors of AEICs cells (Fehrenbach 2011; Williams, 2003).

At the other side of the alveolar epithelium, a monolayer of endothelial cells constitutes the main cell type of pulmonary capillaries. Epithelial and endothelial cells together with their basement membranes form a barrier which is on average 0.5 μm thick (Burns et al., 2003).

Endothelium, thanks to its capability to orchestrate immune and haemostatic responses (Bhattacharya and Matthay, 2013), plays a pivotal role in respiratory pathological conditions. NPs have been shown to be able to directly induce endothelial dysfunction and to have pro-thrombotic effects (Alfaro-Moreno et al., 2007; Mutlu et al, 2007; Nemmar et al., 2004; Nurkiewicz et al., 2006; Vesterdal et al, 2010). Furthermore, NPs inhalation induces inflammatory processes that can lead to endothelial activation and dysfunction, produced by pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6, TNF-α and IL-8 (Szmitko et al., 2003), and characterized by

increased adhesion molecule expression and monocyte adherence (Ramos-Godinez et al., 2013),

In the last few years, several efforts have been made to develop *in vitro* systems more representative of an ABB. The reasons for these efforts are several. For instance, possible translocation paths have been linked to change in the TJs between lung epithelial cells (Geys et al., 2006). Other studies have suggested that the systemic effects could be related to the communication between the cells that interact with the particles and the endothelium (Alfaro-Moreno et al., 2008; Napierska et al., 2012).

The present study aimed to evaluate endothelial cell activation after exposing pneumocytes to nZnO in a co- and tri-culture of the ABB in an *in vitro* system of the ABB in which lung epithelial cells, which are the first target for inhaled particulates, are co-cultured with endothelial cells, which are responsible for the inflammatory response and recruitment of immune response cells observed *in vivo* Furthermore, THP-1 monocytes were added in the basal side to mimic the presence of immune cells in the blood circulation of the pulmonary capillaries. The functionality of the ABB, the release of pro-inflammatory mediators (IL-6 and IL-8) and of endothelial dysfunction markers (sICAM-1 and sVCAM1) were assessed.

2. Materials and methods

2.1 Preparation of NPs suspensions

ZnO NPs (<50 nm) (Sigma Aldrich, Italy) were weighted and suspended in ultrapure sterile Milli-Q water at the concentration of 8 mg/mL. Suspensions were sonicated for 1 min with the sonicator bath Ultrasonic Soniprep 150 MSE (Sanyo), vortexed for 30 seconds and then diluted in PBS + BSA

(0,1% final concentration; Sigma Aldrich) in order to avoid particles agglomeration and optimize suspension stability. Working concentrations (0, 5, 10, 25, 50, 100 μg/mL) were obtained by adding NP suspensions directly to the culture medium (OptiMEM1X, 1% FBS; M199, 1% FBS; GIBCO).

2.2 Cell cultures

Human pulmonary microvascular endothelial cell line HPMEC-ST1.6R was received from Dr. Ronald E. Unger (Institute of Pathology, Medical University of Mainz, Johannes Gutenberg University, Mainz, Germany) and cultured in CellBIND Surface coated flasks (Corning, Italy) in M199 medium supplemented with 15% FBS (Gibco), 2 mM Glutamax I (Sigma Aldrich), 25 µg/mL sodium heparin (Sigma Aldrich; Italy), 25 µg/mL endothelial cell growth supplements (Sigma Aldrich), and 100 U/100 µg/mL Pen/Strep (Euroclone, Italy) at 37°C, 5% CO2. The human lung adenocarcinoma cell line NCI-H441 (ATCC, USA) was maintained in OptiMEM medium (Gibco) supplemented with 10% FBS and Pen/Strep (100 U/ 100 µg/mL), at 37°C, 5% CO_2.

2.3 In vitro alveolar-capillary barrier models: co-cultures and tri-cultures on Transwell inserts

The co-culture was set up as reported in Bengalli et al. (2013). Briefly, HPMEC-ST1.6R cells (9 x10^4 cells/cm^2) were placed on the lower surface of Transwell filter membranes (polyester; 0,4 μm pore size; Costar) coated with 0.2% gelatine and incubated for 2 h at 37°C and 5% CO_2. The filter membranes were then turned upside down and placed in a 12-well plate

filled with 1,5 mL of complete M199 medium and incubated for additional 24 h. The day after, NCI-H441 (2×10^4 cells/cm^2) cells were seeded on the top surface of the transwell filters and cultured to confluence for 11 to 13 days simultaneously with the HPMEC-ST1.6R seeded on the lower surface. To induce differentiation, the NCI-H441 cell line in co-cultures was treated with 1 μM Dexamethasone (Sigma Aldrich) from day 3 of cultivation. nZnO suspensions (10 and 20 µg/mL) were added to the apical compartment at day 12-13th of co-culture, when the epithelial barrier reached the maximum of transepithelial electrical resistance, and exposed for 24h. For the tri-culture model at the 13^{th} day, before apical exposure to nZnO, $6x10^5$ THP-1 cells were added to the basal compartment of the Transwell inserts. The doses of treatment have been defined accordingly to results obtained on NCI-H441 monoculture experiments used for setting up our ABB model.

2.4 Cell viability assays

Different cytotoxicity assays were performed to screen the effects of nZnO on NCI-H441 and HPMEC-ST1.6R cells monocultures and co-cultures. MTT assay and Alamar Blue tests (alamarBlue® Cell viability Reagent, Catalog nos. DAL1025) were performed according to manufacturers' instructions.

The Alamar Blue Reagent (Life Technologies, Italy) is a non-toxic, water-soluble dye that yields a fluorescent signal and a colorimetric change when incubated with metabolically active cells. The oxidized form resazurin (blue) is reduced to a pink fluorescent dye in the medium by cell activity.

The relative viability [%] related to the control samples (untreated cells) was calculated by:

Cell viability = ($OD_{sample}/OD_{control}$)x100.

The data on cell viability are reported as relative decrease compared to the control, considered as 100% of viable cells. Data are presented as mean±SE of at least three experiments.

2.5 TEER measurements

In order to determine the integrity of the ABB, the transepithelial electrical resistance (TEER) was measured with an EVOM Volt Ohm Meter (World Precision Instruments, Berlin, Germany) equipped with an EndOhm 12 Chamber (World Precision Instruments, Berlin, Germany). After NPs exposure, before TEER measurements, transwell filters were washed with PBS and incubated with complete M199 medium for 10 min in order to avoid alteration in TEER values due to cellular debris and inserts handling. TEER of polyester Transwell filter membranes coated with gelatine 0.2%, without cells, was measured and set as blank. TEER was calculated with the following formula:

TEER= (R_M-R_B)xA

where R_M is the experimental value of cell co- or tri-culture resistance, R_B the experimental value of the blank control and A the surface area of the filter membrane (1,12 cm^2). TEER was measured as $\Omega*cm^2$ and values were expressed as % fold increase over the control. The mean ± SE of at least three independent experiments was presented.

2.6 ***Cytokines release***

At the end of the exposure cell culture media were collected and centrifuged at 200xg for 5 min to remove cell debris. The final supernatants were stored at −80°C. IL-6, and IL-8 protein levels were determined by sandwich ELISA (Human Cytoset IL-6 and IL-8; Biolegend) according to the manufacturer's instructions. Absorbance was measured and quantified by a plate reader (Multiskan Ascent, Thermo Scientific Instruments) at wavelengths of 450 and 570 nm, and data expressed as fold increase or pg/ml.

2.7 Release of endothelial damage markers

At the end of the exposure time cell culture media were collected and centrifuged at 200xg for 5 min to remove cell debris. The final supernatants were stored at −80∘C. Soluble form of ICAM-1 and VCAM-1 (sICAM-1 and sVCAM-1) were determined by sandwich ELISA (Life tehcnologies, Italy) according to the manufacturer's instructions. Absorbance was measured and quantified by a plate reader (Multiskan Ascent, Thermo Scientific Instruments) at wavelengths of 450 and 570 nm, and data expressed as pg/ml.

2.8 Co-cultures of endothelial and monocytes cells: modulation of-sVCAM-1 release

HPMEC-ST1.6R cells were seeded in 12-well plates at the density of $10x10^5$ cells/cm^2 in M199 complete medium (15% FBS, Gibco) and incubated at 37°C, 5% CO_2. When endothelial monolayers reached the confluence (4 days), THP-1 cells ($6x10^5$ cells/cm^2) were added in co-culture

with and without sICAM-1 (500 pg/ml) (soluble recombinant ICAM-1, Sigma Aldrich, Italy) and incubated for further 24h.

After that period supernatants from HPMEC-ST1.6R cells alone (C), HPMEC-ST1.6R cells plus sICAM-1, HPMEC-ST1.6R cells plus monocytes (THP-1), and HPMEC-ST1.6R cells plus THP-1 and sICAM-1 (THP-1+sICAM-1) were collected, centrifuged at 200xg for 6 min and stored at -80°C until use for sVCAM-1 ELISA test (Sigma Aldrich, Italy).

2.9 Statistical analysis.

All the experiments were performed in independent triplicates, and data are reported as mean ± SE, if not otherwise specified. Statistical analyses were performed with SigmaStat3.1 software with ANOVA test and post hoc analysis.

3. Results

3.1 Cell viability of monocultures of epithelial and endothelial lines after nZnO

After the exposure 0, 1, 5, 10, 15, 20, 25, 50 µg/ml doses of nZnO there was mortality of 20% in NCI-H441 cells starting from 20 µg/ml, while in HPMEC-ST1.6R cells cell mortality increased at 15 µg/ml.

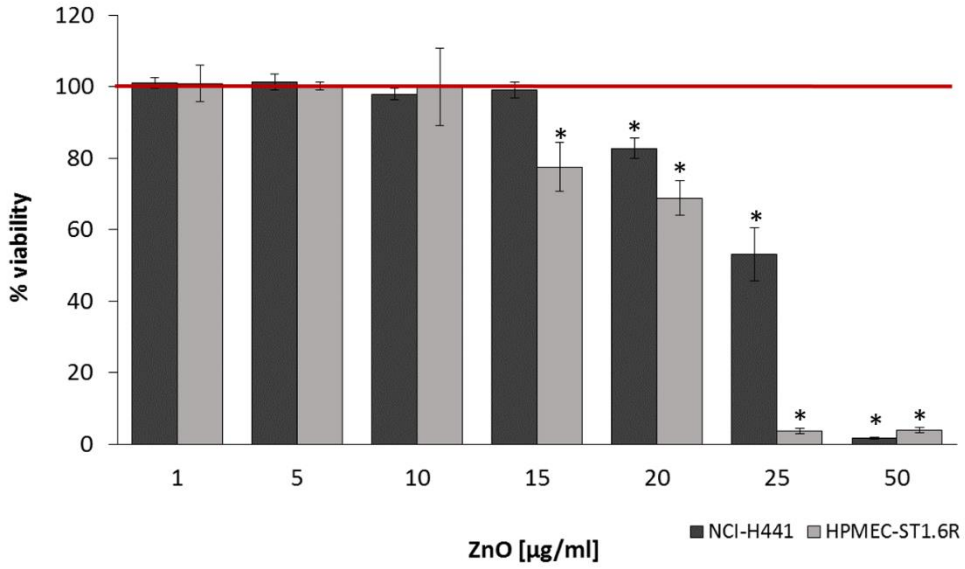


Fig. 1. Effects of nZnO on cells viability. Cells viability assay (MTT assay) in NCI-H441 (black) and HPMEC-ST1.6R cells (grey) exposed to nZnO from 1 to 50 µg/ml for 24h. The data on cell viability are reported as relative decrease compared to the control (red line), considered as 100% of viable cells. Results are presentated as mean± SE of at least three independent experiments. *Statistically different from control (ANOVA, Dunnett's, $p<0.001$).

3.2 Cell viability of co- and tri-culture on Transwell inserts after nZnO exposure

The cytotoxicity of nZnO in co- and tri-cultures was assessed by Alamar Blue. The apical sides (alveolar epithelial cells) of the ABB was treated with 10 (no effect dose in monocultures) and 20 µg/ml (low effece dose) nZnO for 24h and then viability was tested in both compartments. We did not

observed a decrease in cell viability in both co- and tri-culture systems in accordance with the TEER measurements (see Section below).

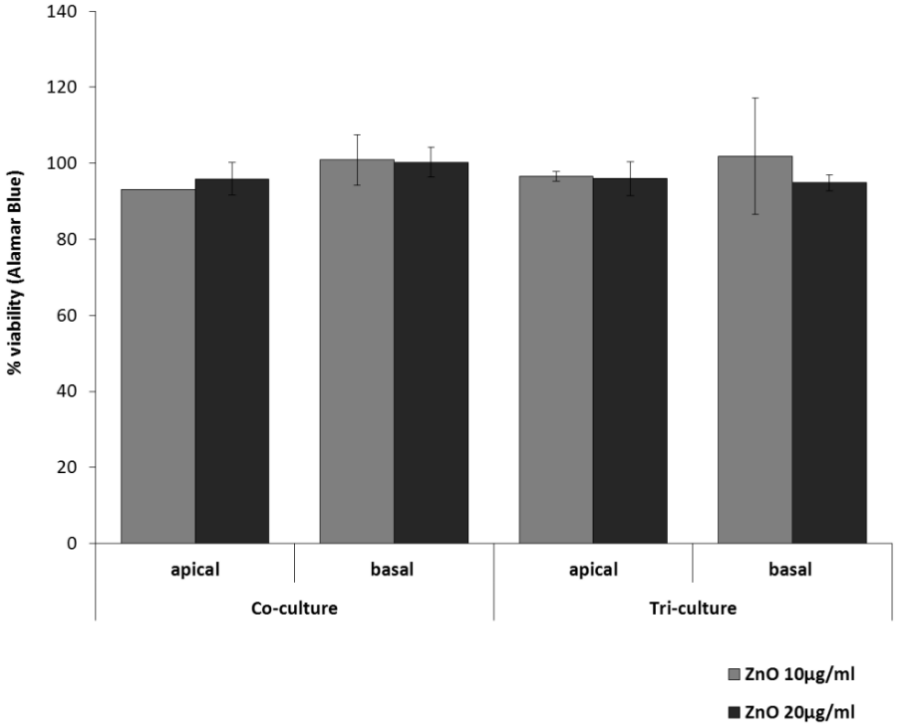


Fig. 2. Cell viability assay (Alamar Blue test) in co- and tri-cultures exposed to nZnO (10 and 20 µg/ml) for 24h. The data on cell viability of the apical compartment (epithelial cells) and of the basal compartment (endothelial cells in the co-culture and endothelial cells plus monocytes in the tri-culture) are reported as relative decrease compared to the control, considered as 100% of viable cells. Results are presented as mean± SE of at least three independent experiments.

3.3 Integrity of the air-blood barrier after nZnO exposure: TEER values

The integrity of the *in vitro* alveolar barrier was assessed by measuring the transepithelial electric resistance (TEER), between the co- and tri-cultures. TEER is an index of the barrier integrity, since it reflects the formation of

tight junction between cells (Hermanns et al, 2004). After 11-13 days, the co-culture model reached a TEER representative of a functional barrier with a maximum of 703 ± 118 $\Omega*cm^2$, in agreement with previous reports (Kasper et al, 2011; Hermanns et al 2004; Bengalli et al., 2013). nZnO (10 and 20 µg/ml) were tested in order to investigate their ability to impair the barrier integrity, evaluated as reduction of TEER values. Data showed that the treatments with nZnO (10 and 20 µg/ml) did not produce reduction of the TEER values in both co- and tri-cultures (Fig. 3). The dose of 100 µg/ml of nZnO was used as highest dose in co-culture condition to evaluate the capability of the assay to measure TEER reduction.

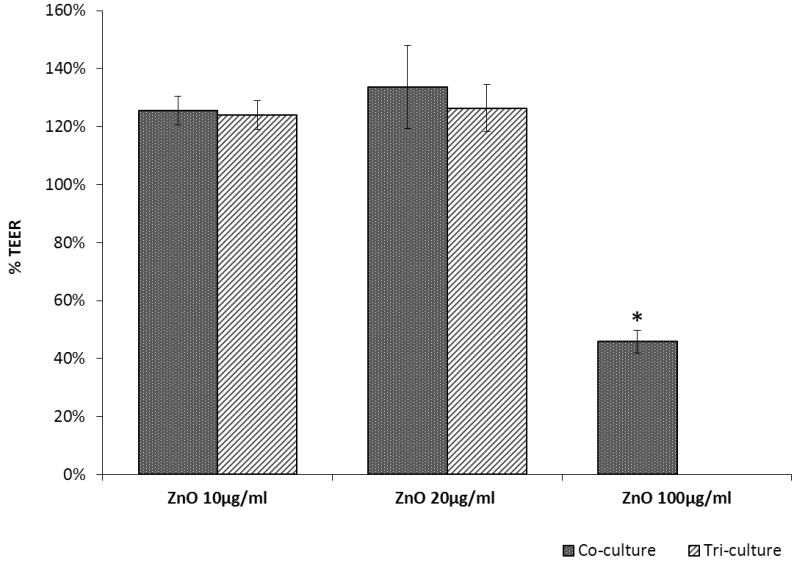


Fig. 3. TEER values percentage respect the control values in co- and tri-culture. The dose of 100 µg/ml of nZnO was used as positive control. Data are expressed as % TEER respect to the control (100%) and represent the means ± SE of three independent experiments. ∗Statistically different from control ($p<0,05$).

3.4 Inflammatory response of epithelial and endothelial cells monocultures to nZnO

Monocultures of epithelial, NCI-H441, and endothelial, HPMEC-ST1.6R, cells were exposed to increasing doses of nZnO (1-25 µg/ml) in order to evaluate the pro-inflammatory potential of NPs. Pro-inflammatory interleukin IL-6 and IL-8 were assessed. The release of IL-6 (control value 23 pg/ml) was significantly increased in NCI-H441 at the dose of 25 µg/ml with a fold increase of 4 time over controls (Fig. 4). IL-8 (control value 271 pg/ml) release was significantly increased after the exposure of 20 µg/ml of nZnO.

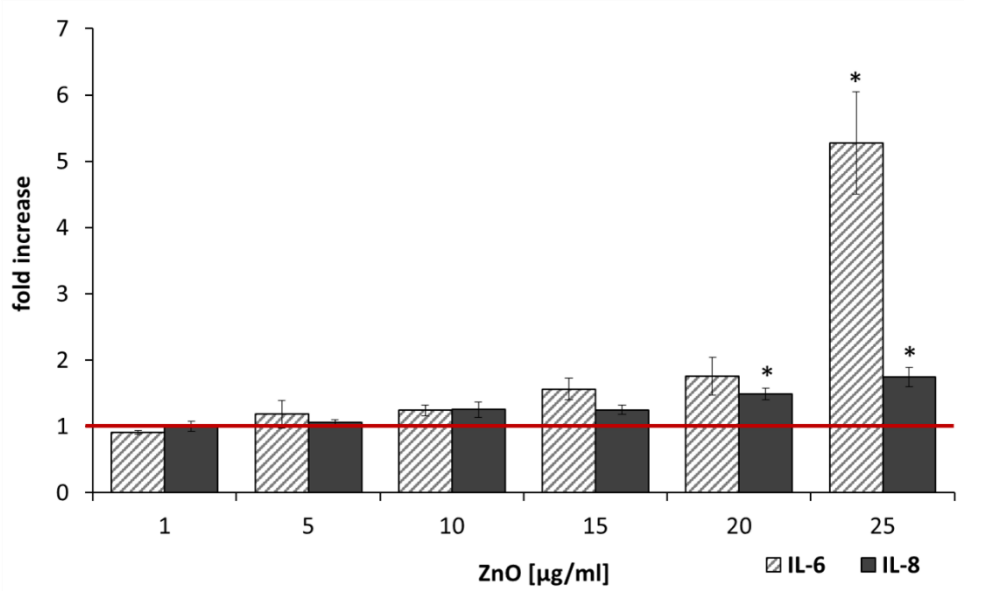


Fig 4. Release of IL-6 (grey bars) and IL-8 (black bars) from monocultures of epithelial alveolar cells NCI-H441 after 24h exposure to nZnO (0-25 µg/ml). Results are presented as fold increase over the control, indicated by the red line. Data are expressed as means ± SE of at least three independent experiments. ∗Statistically different from control(ANOVA, $p<0,05$).

In control samples of HPMEC-ST1.6R the release of IL-6 was of 89 pg/ml, and modulation of IL-6 release was not observed at any concentration of nZnO. The release of IL-8 (control value 164 pg/ml) was significantly increased already at the dose of 10 µg/ml, although a dose-dependent release was not observed (mean increase over control cells of 2 fold) (Fig. 5).

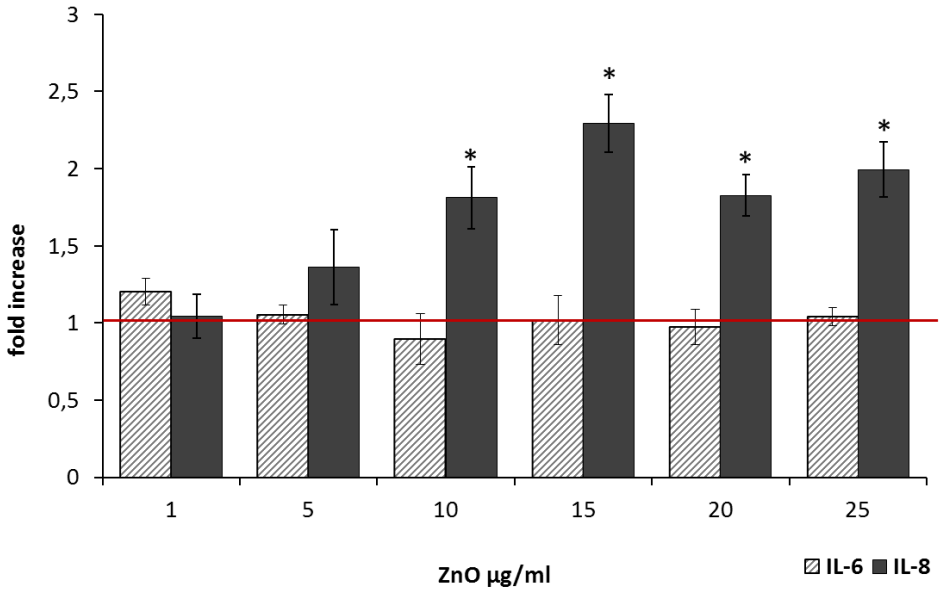


Fig 5. Release of IL-6 (grey bars) and IL-8 (black bars) from monocultures of endothelial pulmonary cells HPMEC-ST1.6R after 24h exposure to nZnO (1-25 µg/ml). Results are presented as fold increase over the control, indicated by the red line. Data are expressed as means ± SE of at least three independent experiments. ∗Statistically different from control (ANOVA, $p<0,05$).

3.5 Inflammatory response of the ABB co- and tri-culture models to nZnO

Co-culture and tri-culture models, at day 13 of culture, were apically exposed to nZnO (10 and 20 µg/ml). The release of pro-inflammatory IL-6

and IL-8, in both apical (alveolar epithelial cells) and basal (endothelial cells) compartments, was evaluate after 24h of exposure. The release of IL-6 from the apical side (epithelial cells) of the co-culture was significantly decreased after nZnO exposure (10 and 20 µg/ml).

The ABB co-culture treated with nZnO (10 µg/ml) showed a significant ($p<0,05$) increase in the release of IL-6 from the basal compartment (endothelial cells) (67 pg/ml of IL-6 in treated cells supernatants versus 10 pg/ml in control ones). The release of IL-6 at the dose of 20 µg/ml was lower respect to the dose 10 µg/ml (22 pg/ml versus 67 pg/ml respectively), but the release was significant respect to the control ($p<0,05$). In treated samples (10 and 20 µg/ml nZnO) the levels of IL-6 released from the basal side are significantly different ($p<0,05$) from the ones released from the apical side at the same dose (18 and 26 fold respectively).In the tri-culture system any release of IL-6 was observed in both compartments (data not shown).

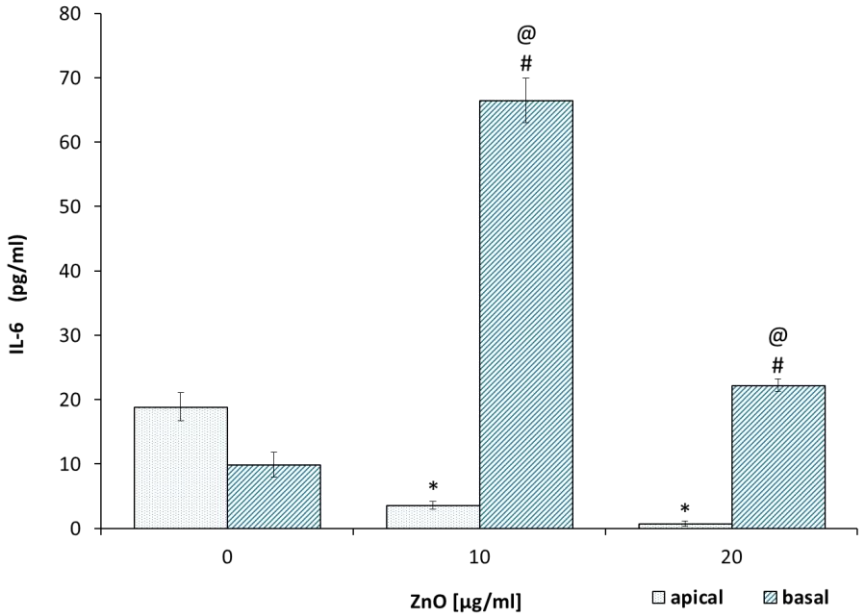


Fig 7. IL-6 release from the co-culture model exposed to nZnO (10 and 20 µg/ml) at day 13 of culture. After apical exposure of the system nZnO the release of IL-6 is significantly decreased in the apical level (epithelial cells) and increased in the basal (endothelial cells) level of the co-culture, after apical exposure. Data are expressed as means ± SE of three different experiments. ∗Statistically different from apical control, @ Statistically different from basal control, # Statistically different from the apical value at the same dose (ANOVA, Dunnett's; $p<0,05$).

In the co-culture system we observed significant release of IL-8 (260 pg/ml of IL-8 in treated cells supernatants versus 96 pg/ml in control ones) from the apical side of the model at the dose of 20 µg/ml of nZnO, while the dose 10 µg/ml (Fig. 8) was uneffective. The ABB tri-culture treated with 10 µg/ml nZnO showed a significant release of IL-8 from both apical (96 pg/ml of IL-8 in treated cells supernatants versus 54 pg/ml in control ones) and

basal (173,5 pg/ml of IL-8 in treated cells supernatants versus 63 pg/ml in control ones) compartments (Fig. 9). The amount of IL-8 released from the basal 1,8 fold higher respect to the apical one. Interestingly, in absence of THP-1 in the basal compartment (co-culture system), the release of IL-8 is not modulated (Fig. 8).

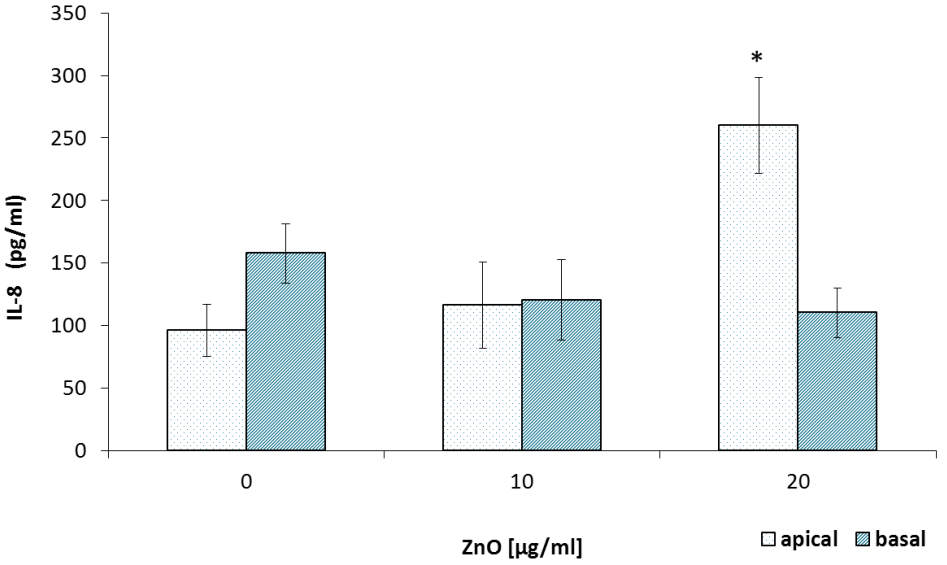


Fig 8. IL-8 release from the co-culture model exposed to nZnO at day 13 of culture. At the dose of 20 µg/ml of nZnO the release of IL-8 is significantly increased in the apical side (white bars) of the co-culture, but not in the basal compartment (light blue bars). Data are expressed as means ± SE of 3 independent experiments. ∗Statistically different from apical control (ANOVA, Dunnett's; $p<0,05$).

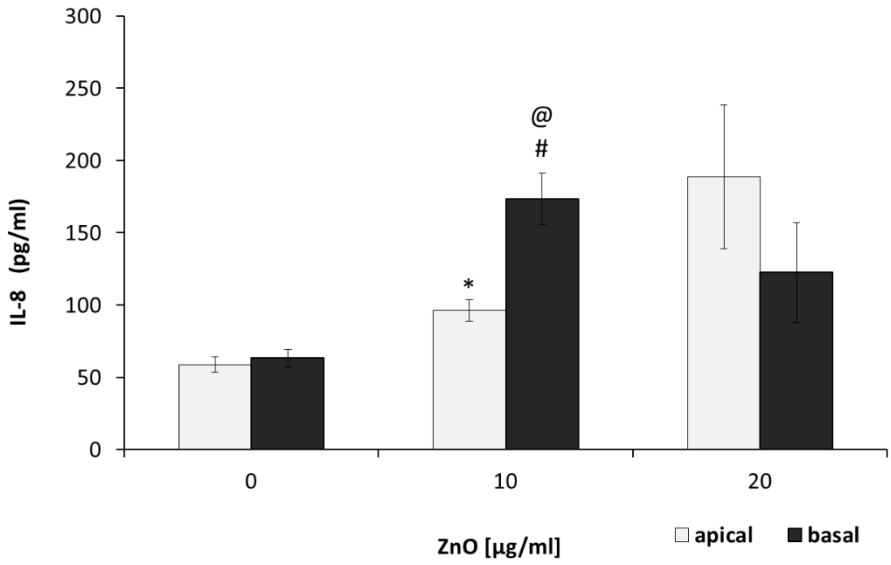


Fig 9. IL-8 release from the tri-culture model exposed to nZnO at day 13 of culture. The release of IL-8 is significantly increased in both apical (white bars) and basolateral (black bars) sides of the tri-culture at the dose of 10 µg/ml. In the presence of THP-1 cells on the basal side, the release is more relevant in the basal (endothelial cells) compartment of the model after apical exposure of the system (NCI-H441 compartment). Data are expressed as means ± SE of three independent experiments. ∗Statistically different from the apical control, @ Statistically different from the basal control # Statistically different from the apical value (ANOVA, Dunnett's; $p<0,05$).

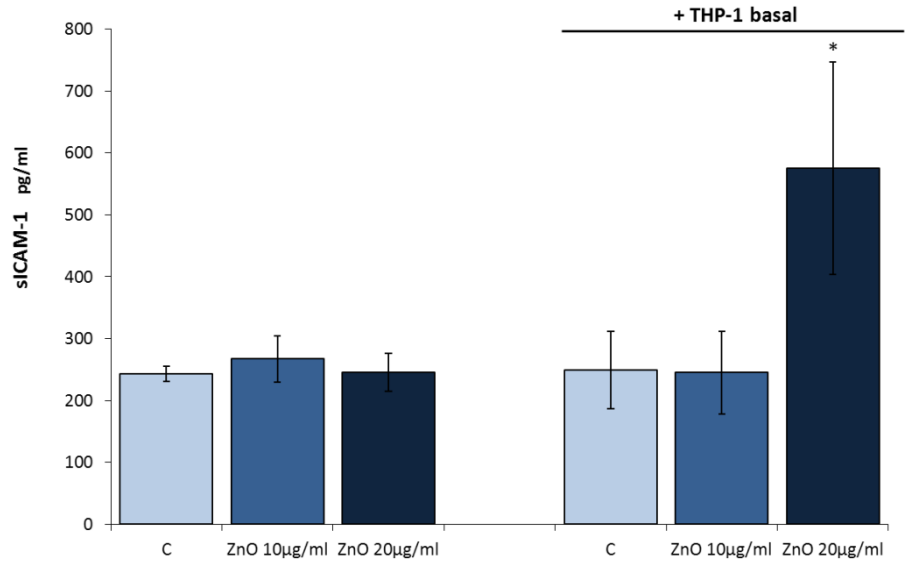
3.6 Release of endothelial markers

The endothelial specific inflammatory markers, soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1), were evaluated in the basal compartment of the co-culture (Fig. 10A) and tri-culture (Fig. 10B). We observed an increase in the basal release of sICAM-1 in presence of basal THP-1 at the dose of 20µg/ml

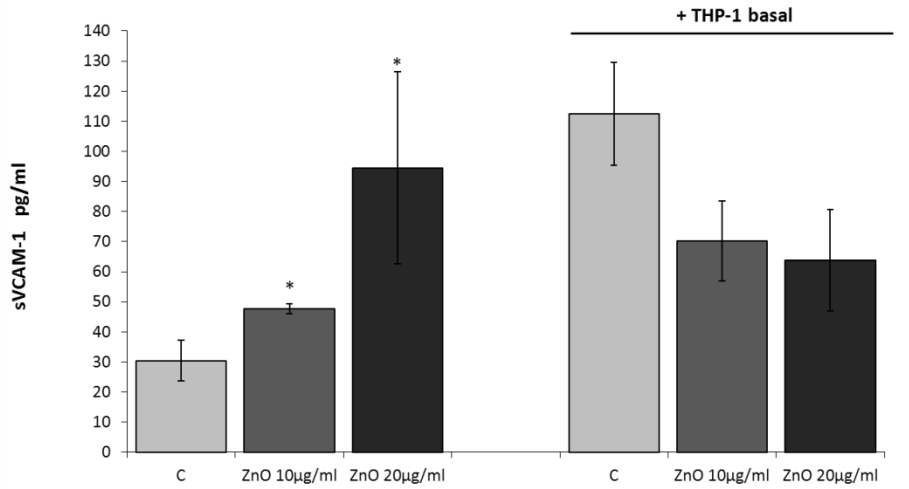
(Fig. 10A), while sVCAM-1 levels increased in the co-culture, but in presence of THP-1 the release is slightly, but not significantly decreased (Fig. 10B).

As observed in figure 10B the presence of THP-1 in the model seems to modulate the release of sVCAM-1 in the basal compartment of the tri-culture. In order to evaluate the ability of THP-1 cells to influence the release of this endothelial marker, we co-cultured monocytes and endothelial cells together and investigated the release of sVCAM-1. The modulation by sICAM-1 to modulate sVCAM-1 release was also evaluated by treating endothelial+THP-1 cells with the maximal amount of sICAM-1 (500pg/ml) observed in the basal compartment of the tri-culture.

In Fig. 11 are presented the values of sVCAM-1 expression obtained by the co-culture of endothelial cells with monocytes. Values from HPMEC-ST1.6R monocultures are also presented as basal expression of sVCAM-1 from endothelial cells. In presence of THP-1 or sICAM, the release of sVCAM-11 in the culture medium seems to be enhanced, while, when THP-1 were treated with sICAM-1, we observed that sVCAM-1 release is comparable to control values. This situation was similar to the one observed in the basal compartment of a tri-culture model apically exposed to nZnO, in which sVCAM-1 release is decreased and sICAM-1 levels are enhanced by monocytes. These data suggest that sVCAM-1 expression in the co-culture of endothelial cells and THP-1 cells is in part regulated by monocytes and sICAM-1 stimulation.



A



B

Fig 10. Release of vascular endothelial damage markers at 24h of exposure to 10 and 20 µg/ml nZnO. The endothelial specific inflammatory markers, soluble intercellular adhesion molecule-1 (sICAM-1) and vascular cell adhesion molecule-1 s(VCAM-1) have been evaluated in the basal compartment (Fig. 10A,B). *Significantly different from the control (ANOVA; $p<0,05$).

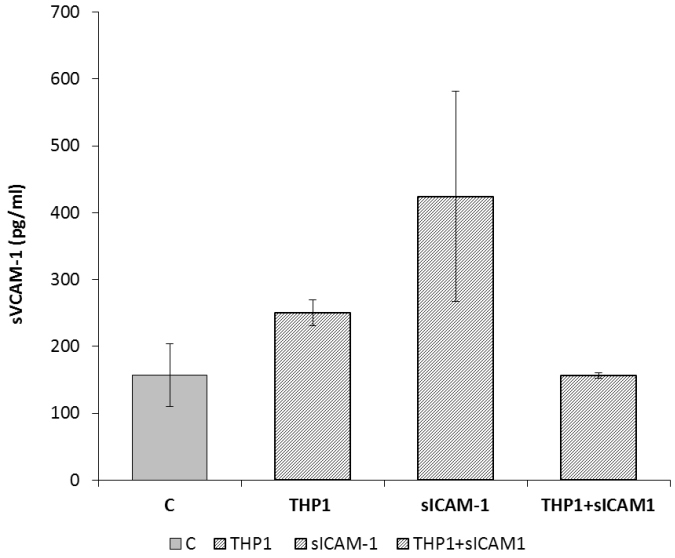


Fig. 11. Release of sVCAM-1 from monocultures of endothelial cells without (C) and with (sICAM-1) the presence of sICAM-1 in the culture medium and from co-cultures of endothelial cells and monocytes without (THP-1) and with (THP-1+sICAM-1) sICAM-1 in the medium.

4. Discussion

4.1 Effect of nZnO on the ABB integrity

Epidemiological and experimental *in vivo* studies have associated cardiovascular impairments with pulmonary deposition of NPs. Clinical data show that high concentration of UFP (particles smaller than 100 nm) in the ambient air can cause several health effects, both at the respiratory level and cardiovascular system (Nemmar et al., 2006; Geiser et al., 2005). NPs and UFP, for their small dimensions, are able to cross ABB and pass into the blood stream where they can interact with cells of the immune system, affect the vascular endothelium and may reach secondary target organs. Nevertheless, it is still unknown the fraction of inhaled particles that cross

the ABB and pass into the circulation and therefore the amount of NPs that are able to interact with endothelial cells has still to be determined. Studies on rats suggest that percentage of NPs translocating to the blood stream is about 1% of particles deposited in the lungs (Kreyling et al., 2002) and that only the 0,1% is able to reach secondary organs in which they may accumulate (Kreyling et al., 2009). Furthermore according to Geiser and colleagues (2005) the amount of titanium oxide NPs (TiO_2) that can reach the vascular compartment is about the 10-15% of the inhaled one. Cohen et al. (2014) developed an *in vitro* model for tracking industrial relevant metal oxide NPs/ENMs across a cellular monolayer. They demonstrated that nZnO, SiO_2-coated CeO_2 and SiO_2-coated-ZnO NMs were able to translocate across lung epithelial cell monolayers (Calu-3 cell line) at low doses (12,5 µg/ml), without affecting the trans-epithelial electrical resistance (TEER), that means without compromising the alveolar epithelial barrier integrity. Among the three NPs, nZnO exhibited the greatest amount of translocation following 24h exposure and the tendency to form agglomerates of 230nm as determined by DLS analysis in the basolateral media of the *in vitro* model (Cohen et al., 2014). These finding support the capability of MONs to cross the ABB, affecting directly also the vascular endothelium (Gojova et al., 2007; Sun et al., 2011). In our ABB *in vitro* models we have not observed the translocation of NPs (data not shown). However it is important to notice that in our experiment we used Transwell inserts with pores of 0,4, µm in order to reach an high TEER with NCI-H441, while in Cohen's study the inserts used were with pores of 3 µm with Calu-3 cells which are bronchial cells with an higher TEER. This difference

can explain the discrepancy observed and influence the ability of NPs to reach the basal side in a Transwell system (Geys et al., 2006).

In our study the TEER of the ABB models exposed to nZnO was not affected by the treatments, confirming that the low doses (10 and 20 μg/ml) used did not induce significant cell toxicity in the epithelial cell line when co-cultured with endothelial cells. These data were also supported by the viability test Alamar Blue that demonstrates that apical exposure to nZnO did not affected cell viability in both apical and basal compartments of the system.

Previous work from our laboratory showed that also ABB models exposed to TiO_2 NPs or to PM10 (25 μg/ml for 24h) showed no differences with respect to the controls (Bengalli et al., 2013). On the contrary copper oxide NPs (nCuO) induced significant effects on TEER, therefore confirming the ability of the NPs to affect the functionality of the ABB. nZnO resulted to be more toxic and able to affect TEER in rat alveolar cells monolayers and that was partly related to the dissolution of Zn^{2+} ions from the particles (Kim et al., 2010). The differences in TEER reduction may depend by the specific cell line, which may have a different sensitivity to nZnO and also depend by the presence endothelial cells in the basal compartment of the Transwell. It is well known that endothelial cells have a crucial role in maintaining the barrier homeostasis and functionality (Bhattacharya and Matthay, 2013). Furthermore in Kim and co-workers study (2010). TEER-reduction occurred after exposure to 176 μg/ml, an extremely high concentration of nZnO, while we exposed cells to 10 and 20 μg/ml, low doses.

It has been reported (Kasper et al., 2011) that co-culture systems are less sensitive to NPs-induced cytotoxicity, therefore, these results here observed could be related to an overall lower responsiveness of the ABB model to the NPs used.

4.2 Inflammation and endothelial dysfunction caused by nZnO

Many experimental evidences have demonstrated that lung exposure to metal oxide NPs, such as nZnO, cause pulmonary and vascular inflammation (Gojova et al., 2007; Tsou et al., 2010; Han et al., 2013). Once inhaled the main target of NPs are the alveolar epithelial cells, but at the ABB level different cell types communicate by direct contact or release of biochemical mediators in order to orchestrate the functions of the respiratory barrier. The vascular endothelium plays a central role in the inflammatory process and cytokines production and NPs can stimulate endothelial cells by direct contact after their translocation through the alveolar epithelium or by inducing release of inflammatory mediators in the alveolar space (Mills et al., 2009; Nel et al., 2006). However, *in vivo* data from rats have shown that the amount of NPs at the alveoli able to translocate through the ABB is very small (1%) (Kreyling et al., 2002), suggesting that the release of mediators, such as cytokines and endothelial dysfunction markers, triggered by NPs exposure, could be the major event involved in vascular inflammation and cardiovascular diseases progression.

The *in vitro* model developed in this study, in which epithelial (apical) and endothelial cells (basal) co-exist on a Transwell inserts, allows the exposure of epithelial cells to NPs and the detection of inflammatory mediators as well as other markers of endothelial activation in the cells underlying the

epithelial layer. Furthermore, the addition to this system of a cell line derived from the immune system, such as monocytic cell line, could be a useful method to evaluate also the modulation of the immune system to the biological responses induced by NPs.

The major pro-inflammatory mediators released after NPs exposure are IL-8, IL-6, IL-1β and TNF-α (Nemmar et al., 2013).

IL-6 is a pleyotropic cytokine with a complex role in the modulation of the immune and inflammatory responses, such as the induction of B cells differentiation, the expression of acute phase proteins and the recruitment of leukocytes by up-regulating chemokines production and adhesion molecules expression (Oh et al., 1998; Romano et al., 1997; Modur et al., 1997).

IL-8 is a pro-inflammatory protein mainly derived from epithelial cells and in pulmonary inflammation and it plays an important role in mediating pathogenesis in the lung (Standiford et al., 1993; Allen et al., 2014).

Previous works have demonstrated that *in vitro* cells exposure to nZnO induces inflammatory response determined by the release of interleukins, such as IL-8 (Gojova et al., 2007; Sun et al., 2011) and IL-6 (Roy et al., 2014).

In our study we investigated the expression of IL-6 and IL-8 in both apical and basal compartments of the co- and tri-culture systems after apical exposure to nZnO in order to evaluate nZnO potential effects on endothelial inflammation. The treatment with 10 µg/ml nZnO induced release of IL-6 in the basal compartment of the co-culture system while we did not observed release of the cytokine in the tri-culture system. On the contrary nZnO did not induced IL-8 secretion in the culture medium of the basal side, but in presence of monocytes (tri-culture) IL-8 level are increased in both apical

and basal compartments, with higher expression in the last one. These data evidenced that the apical exposure to nZnO can induce not only a direct local inflammatory effect in epithelial cells, but they can also activate endothelial cells inducing the secretion of pro-inflammatory mediators. Furthermore the presence of cells of the immune systems seems to modulate the release of the cytokines, inhibiting the release of IL-6. This must be explained by the high expression on monocytes of the IL-6 receptor (IL-6R) (Wognum et al., 1993), consistent with a possible direct biologic effect of IL-6 on these cell types. On the contrary the co-presence of monocytes and endothelial cells determined an increased expression of IL-8. This is in accordance with results from Liu and co-workers (2012) that showed the importance of cell-to-cell contact as a prerequisite for enhanced cytokine generation after particles stimulation.

Furthermore our data agree with previous works in which the presence of different cell types in in co- and tri-cultures and the consequent cellular communication enhanced the inflammatory response after exposure to particles (Alfaro-Moreno et al., 2008) and NPs (Kasper et al., 2011).

Since the expression of cytokines mediates the adhesion of monocytes to the endothelium, the overexpression of these mediators can lead to an enhanced interaction between these cell types increasing monocytes migration and promoting foam cells formation, a hallmark of atherosclerosis (Suzuki et al., 2014).

Another event that can occur after NPs exposure is endothelial cell activation and dysfunction. This phenomenon is characterized by an up-regulated expression of cellular adhesion molecules (CAMs), such as ICAM-1 and VCAM-1, on endothelial cells. ICAM-1 is the ligand for LFA-

1 (CD11a/CD18) and Mac-1 (CD11b/CD18) on granulocytes and lymphocytes. VCAM-1 is present on activated endothelial cells and serves as a ligand for very late antigen-4 (VLA-4), which is present on leucocytes (Reinhart et al., 2002).

These molecules are expressed at low level in endothelial cells and after stimulation by particles or cytokines, their expression increases promoting leukocytes adhesion to the endothelium (Kayal et al., 1998; Kjærgaard et al., 2013). Yatera and co-workers (2008) have demonstrated that the deposition of particulate in mice lungs is associated with the up-regulation of ICAM-1 and VCAM-1 on the endothelium and development of atherosclerotic plaque, supporting the idea that CAMs are involved in the progression of vascular diseases.

CAMs exist also in a soluble form (sCAMs) and they can be quantified *in vitro* (Kjærgaard et al., 2013). Elevated levels of sCAMs have been reported in numerous diseases, such as infections, inflammation, autoimmune diseases and cancer (Gearing et al., 1992; Gearing et al., 1993).

The increased levels of sCAMs in these diseases have been attributed to the up-regulation of CAMs expression following endothelial activation. Recent works have demonstrated that sCAMs levels correlate with surface expression, making these soluble forms as potential biomarkers of the degree of endothelial activation (Videm et al., 2007; Kjærgaard et al., 2013).

The *in vitro* activation of endothelium after apical exposure to NPs has been previously demonstrated by Ramos-Godinez and co-workers (2012) which observed an enhanced expression of CAMs in a co-culture systems of A549 and HUVEC apically exposed to TiO_2 NPs, although there was not an increase in the levels of inflammatory cytokines neither of sCAMs.

In our study we have investigated the expression of sICAM-1 and sVCAM-1 in the supernatants from endothelial cells following the apical exposure to nZnO in both co- and tri-culture systems. We observed that nZnO induced an increase in release of sVCAM-1 from the basal compartment of the co-culture and a decrease of sVCAM-1 in the tri-culture systems. For sICAM-1 we observed no induction by nZnO in the co-culture and an increase in the tri-culture at the highest dose of exposure (20 µg/ml).

The augmented release of sVCAM-1 in the co-culture indicates that after NPs exposure endothelial dysfunction occurs. Furthermore the *in vitro* expression of sCAMs can be modulated not only by the stimulus used, but also by cytokines produced by the endothelial cells themselves (Schildberger et al., 2010). The absence of modulation of sVCAM-1 in supernatants of tri-culture samples may be explained by the lack of cytokines release as well as by the presence of monocytes and their enhanced adhesion to endothelial cells (Jacobson et al., 2000).

The presence of monocytes in our system upregulated the release of sICAM-1 in the basal side after nZnO exposure. Furthermore in supernatants of control samples of the tri-culture the expression of sVCAM-1 was higher respect to the sVCAM-1 expression in control samples of the co-culture. We supposed that the presence of sICAM-1 in the basal compartment of the system might regulate the release of sVCAM-1. In order to support this hypothesis we have co-cultured endothelial cells and monocytes and analysed the expression of sVCAM-1 in the absence and presence of sICAM-1, by exposing cells to amount of sICAM-1 comparable to the one detected in the medium of the tri-culture samples (500 pg/ml). We have observed that the expression of sVCAM-1 seems to be increased in

presence of THP-1, but by exposing the co-culture to sICAM-1 sVCAM-1 values are decreased, supporting our hypothesis of a modulation of the expression of sCAMs in the presence of monocytes in culture with endothelial cells.

Further investigation however is needed to clarify these findings, since, the expression of CAMs strictly depends on the presence of different cytokines, including TNF-α, IL-1β and IL-4 and IL-10 which can influence the expression of a specific CAM (Daxecker et al., 2002).

These data demonstrated that the exposure to nZnO induces the release from endothelial cells of both inflammatory and endothelial dysfunction markers, which can be correlated to the onset of pulmonary and cardiovascular diseases as well as systemic effects observed in *in vivo* experiments.

5. Conclusions

The development of nanotechnology is arising over the years increasing concern about the emission of NPs in the environment and subsequent unintentional human exposure. The high quantity of NPs need to be screened in order to understand their biological response and to prevent health hazard. *In vivo* systems are largely used but since the demand on elucidating NPs effects is increasing drastically there is an urgent need to develop and test NPs on alternative *in vitro* methods, both for scientific and ethical issues. The cultivation of cells in co- and tri-cultures are *in vitro* methods that more realistically represent the complexity of a biological apparatus, such as a biological barrier, mimicking a situation more similar to the *in vivo* one. Thus, they are suitable systems for studying the toxicological events induced by NPs at the alveolar level.

Our models showed that the exposure to nZnO is not able to impair the functionality of the barrier, but induces the activation of the endothelial cells by the release of inflammatory mediators (IL-6 and IL-8) and endothelial dysfunction markers (sICAM-1 and sVCAM-1), even if NPs are not in direct contact with endothelial cells. Furthermore, the presence of cells from immune system (monocytes) were able to modulate the response to nZnO. In conclusion, although the molecular mechanisms need to be clarified, the *in vitro* systems here presented offer an important insight of the key role of the communication among different cell types. Furthermore these systems seem to be good candidates in order to have a more complete overview of the events that may occur at the alveolar region after NPs inhalation, especially when their ability to induce cardiovascular and immune responses are considered.

Further studies will be helpful to have a better knowledge of the molecules responsible for the cellular crosstalk and to delineate a more complete cytokine and sCAM panel able to regulate pulmonary and endothelial dysfunction and inflammation. Moreover the adhesion of monocytes to endothelial cells will be also another mechanism to be investigated since a possible correlation between NPs exposure and an enhanced adhesion of these cells will consent to understand the NPs-induced vascular effects, such as the developing of atherosclerotic plaques.

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CHAPTER VI

Exposure of *in vitro* models of the respiratory barrier to the Air Liquid Interface: role of nanoparticles system of exposure

Abstract

Several studies confirm that the inhalation of nZnO causes respiratory and systemic effects. There are also evidences demonstrating that different biological NPs-induced effects may depend on the different modality of their administration to cultured cells. Since the respiratory epithelium functions are orchestrated by the interplay of several cell types there is a need for *in vitro* models to study the mechanisms underlying inhaled NPs biological effects under different exposure conditions. An *in vitro* model consisting of a Transwell co-culture of murine alveolar epithelial cells (AECs) and alveolar macrophages (AMs) was exposed to nZnO in submerged and Air-Liquid Interface (ALI) conditions. nZnO cytotoxicity was assessed and inflammatory and oxidative stress genes expression were investigated in the co-culture model upon both exposure conditions. The expression of genes involved in inflammation (*Il1b*, *Tnf*) and oxidative stress (*Hmox1*) were regulated after nZnO exposure. Moreover, data showed that the cytotoxic effect of nZnO was higher in the murine co-culture exposed at the ALI compared to the submerged conditions. These results confirm that *in vitro* co-culture models of the respiratory apparatus are useful tools for understanding NPs-induced effects in studies of inflammatory processes in which different cell types and exposure conditions contribute to promote the biological outcomes.

1. Introduction

Nanotechnology is developing rapidly and several 'nano-objects' are already present in consumers products. According to this evidence, there is a great concern about NPs toxic effects and the risk associated to their exposure. NPs may become airborne during manufacturing, packaging and application, by handling during waste incineration and accidents (Paur et al., 2008). NPs in the airborne can reach the lungs, which are, with their extension of 150 m^2, the largest surface exposed to the ambient air. Thanks to their numerous application in several fields, in the last decades the production of nZnO is exponentially increased in daily-life goods, leading to enhanced emissions in the environment and in workplaces. Zinc is a ubiquitous transition essential metal associated to industrial emissions and it typically appears in the form of ZnO in ambient particulate matter (PM) (Adamson et al., 2000; Councell et al., 2004). nZnO are known as an occupational hazard and their inhalation can cause systemic effects (e.g. metal fume fever) and inflammatory status (Chen et al., 2014). When entering the respiratory tree NPs can reach the distal region of the respiratory epithelium, the alveoli, where there are different cell types: alveolar macrophages (AMs), alveolar epithelial cells (AECs) and endothelial cells (ECs). Since the respiratory epithelium is complex and its functions are orchestrated by the interplay of different cells there is a need of *in vitro* complex systems that mimic the ABB in order to clarify the mechanisms underlying NPs toxicity and their biological effects.

Co-culture *in vitro* systems are useful tools that consider the intercellular communication between different cell types of the lung. Since the inflammation is one of the major responses involved in nZnO exposure (Xia

et al., 2008; Roy et al., 2014a), the addiction of immune cells to culture systems is of great importance when studying inflammatory effects upon nanoparticles exposure. A co-culture system composed of AECs and AMs simulates the *in vivo* cross-talk that occurs between these different cells in order to coordinate immune response (Paur et al., 2011, Roggen et al., 2006).

Another important aspect to take into account when considering NPs testing is the way of particles administration. Actual *in vitro* assays disperse NPs in cell culture medium and then expose *in vitro* models to this suspension. This protocol, although widely used in *in vitro* toxicological studies, has some main limitations. First, submerged exposure and cell culturing represents unrealistic exposure scenario for lung cells. Lung epithelial cells *in vivo* grow and come in contact with pollutants in an air-liquid interface (ALI), therefore an *in vitro* system able to mimic this situation could be more realistic than systems in which cells are immersed in culture medium (Lenz et al., 2009). Second, the exposure of airborne nanoparticles at the ALI can avoid the aggregation of NPs that occurs when suspended in culture medium (Paur et al., 2011), although the fate of NPs in the lungs (in which high content of humidity and presence of biological macromolecule, surfactants, are present) is not completely clear.

Finally, in submerged conditions the particle dose interacting with the cells is often unknown, especially for small particles, when diffusion becomes the dominant transport mechanism, leading to particles dispersion toward lateral walls (Shaw 1992).

Although many researches are focused on the development of *in vitro* methods to study the dosimetry of delivered particles, data on the different

effects caused by NPs in submerged or ALI conditions are still lacking (Lenz et al., 2013).

The aim of this paper is to evaluate the differences in toxicity of nZnO in an *in vitro* model of the respiratory barrier/alveolar space with different exposure conditions: submerged and ALI. Cell viability, morphological changes and the expression of genes involved in oxidative stress (*Hmox1*) and inflammation (*Tnf* and *Il1b*) have been analyzed in order to investigate the mechanisms underlying nZnO-induced effects.

2. Material and Methods

2.1 Particles

Zinc oxide nanoparticles (nZnO) were obtained from Sigma Aldrich with a nominal average diameter of 50 nm. nZnO suspensions were prepared in sterile, double-distilled water. Stock solution (8 mg/ml) were sonicated for 15 min prior to serial dilutions, and each suspension was sonicated for 10 min directly before use.

2.2 Cell cultures

The murine alveolar epithelial-like type II cells LA-4 (ATCC No. CCL-196) were grown in HAM's F12 medium with stable Glutamax containing 15% foetal bovine serum (FBS, Gibco, Germany) and 1% non-essential aminoacids (NEA, Biochrom AG, Seromed, Germany) and 100U/ml penicillin/streptomycin (Biochrom AG, Seromed, Germany) at 37°C, 5% CO_2. Murine alveolar macrophages MH-S cells (ATCC No. CRL-2019) were grown in RPMI-1640 Medium (Life Tecnologies, Germany) supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol. Cell were

passaged every 2-3 days. LA-4 and MH-S cells were seeded at a density of $2.5x10^4$ cells/well in 96-well plates (BD Falcon, Germany) and were grown overnight in an incubator at 37°C, 5% CO_2. After 24h cell culture medium was replaced and the appropriate amount of particles dilution was added to the well. Cells were treated with nZnO (0, 5, 10, 20, 25, 50 µg/ml) for 4h, 6h and 24h.

2.3 Co-cultures set-up on Transwell inserts

LA-4 were seeded at the density of $0,5x10^6$ cells/insert on the opposite side of a Transwell insert (BD Falcon, transparent PET membrane, effective growth area 4,2 cm^2, 0.4µm pore size, $1.6x10^6$ pores/cm^2) and let to adhere for at least 2h in the incubator at 37°C, 5% CO_2. After that, insert was turn and put in a well of 6-well culture plate for 24h immersed in 3ml of culture medium in the lower compartment and 2ml in the upper compartment. After the cells had formed a confluent monolayer (24h), MH-S cells (in a ratio of 1:4 meaning $0,125x10^6$ cells/inserts) were seeded in the upper compartment for 24h in submerged conditions, at 37°C, 5% CO_2, in order to let them adhere to the insert.

2.4 Co-cultures handling for submerged and ALI exposure

For submerged exposure experiments culture medium was removed from the apical side of the co-cultures and replaced with 2 ml of MH-S serum-free fresh-medium containing 5 µg/ml (corresponding to 10,5 µg/cm^2) of nZnO suspension. The medium in the lower compartment was replaced with 3 ml of LA-4 fresh serum-free medium. Cells were then incubated for 6h at 37°C, 5% CO_2.

For ALI exposure experiments, cells medium was removed from the apical side of the system at least 1h earlier before the exposure to particles. The medium in the basal compartment was replaced with 3 ml fresh medium and cells were put in the exposure chamber in which 5 µg/ml nZnO were nebulized for 3 min (Fig. 1). After that, cells were removed from the chamber and incubated for 6h at 37°C, 5% CO_2. After the post-incubation cells were washed with PBS and used for viability test (WST-1 assay, Roche) and then lysed on the insert membrane by adding 350 µl of lysis buffer suitable for RNA isolation (Qiagen). Data are expressed relative to control conditions (incubated cell cultures without nZnO).

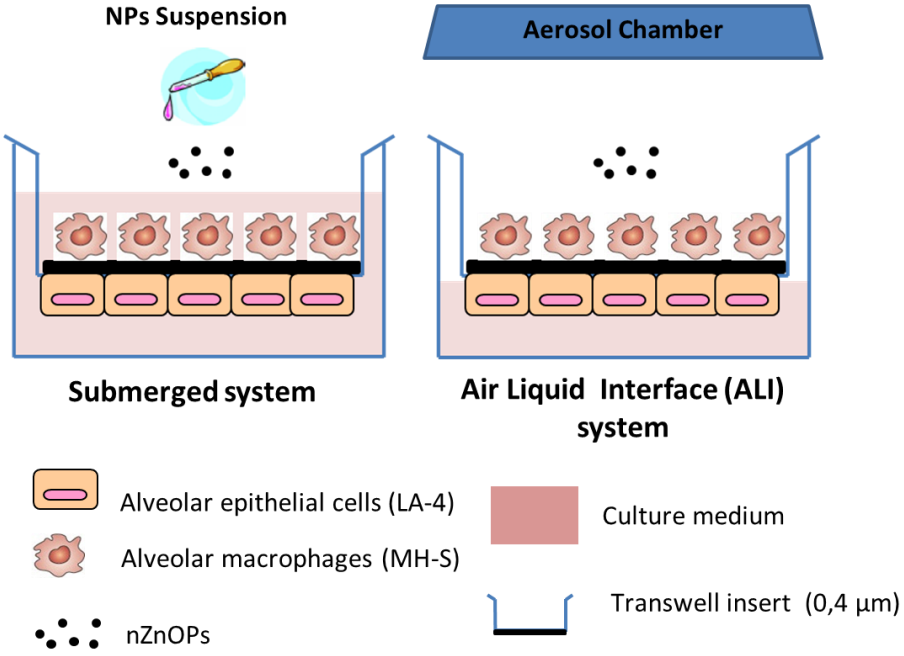


Fig. 1. Schematic representation of the submerged and ALI exposure systems.

2.5 Principle of operation of the ALICE-CLOUD

The ALI exposure consists of an Air–Liquid Interface (ALI) Cell Exposure–Cloud system (ALICE-CLOUD) schematically depicted in Fig. 2.

The ALICE-CLOUD comprises a cuboidal exposure chamber (L×W×H=12.8cm×8.6cm×16.0cm) containing a vibrating mesh cloud generator (Aeroneb Pro, Aerogen 112 Inc., Galway, Ireland) at the center of the ceiling oriented towards a standard multi-well plate at the bottom of the chamber. There is no air flow into or out of the device. A commercial version of this device has been made available by Vitrocell Systems (Waldkirch, Germany; www.vitrocell.com).

The experiment with the ALI systems were performed at the Comprehensive Pneumology Center, Institute of Lung Biology and Disease (iLBD), Helmholtz Zentrum München - German Research Center for Environmental Health, Munich (DE), with the supervising of Dr. Tobias Stöger and Dr. Anke Lenz, who have set up the system (Lenz et al., 2009, Lenz et al., 2014).

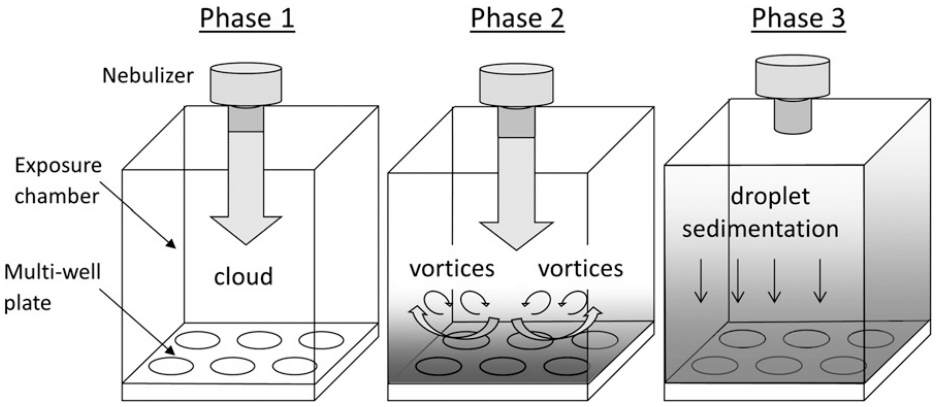


Fig. 2. Experimental setup and principle of operation of the Air–Liquid Interface (ALI) Cell Exposure–Cloud system (ALICE-CLOUD). The ALICE-CLOUD consists of a cuboidal exposure chamber with a nebulizer on top directed toward a multiwell cell culture plate at the bottom. Phases of operation are as follows. Phase 1: nebulizer emits a dense cloud of droplets. Phase 2: emitted cloud is decelerated by air drag and then diverted horizontally in all directions, resulting in convective mixing by vortices, transforming the cloud into a uniformly distributed mist of droplets which fills the chamber from bottom up. Phase 3: droplet sedimentation deposits the uniformly distributed droplets onto the cells. This experimental procedure is performed at room temperature for about 5 min.

2.6 Viability assay

Cell viability was determined using the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Germany). WST-1 is a substrate which measures the metabolic activity of viable cells and the assay is based on the reduction of the WST-1 to a water soluble formazan salt. WST-1 is an analogous form of MTT. The tetrazolium salts are cleaved to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This

augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the culture (Berridge et al., 1996). In contrast to MTT which is cleaved to water-insoluble formazan crystal and therefore has to be solubilized after cleavage (by the addition of DMSO), WST-1 yields water-soluble cleavage products which can be measured without an additional solubilization step. Furthermore WST-1 is less cytotoxic for cells and after the viability test cells can be used for further analysis, such as RNA or protein extraction.

In monoculture experiments, LA-4 and MH-S cells were seeded at the density of $0.25x10^5$ cell/well/cm^2 in 96-well plate in cell culture medium containing FBS and grown overnight in an incubator at 37°C and %% CO_2. For the treatment the medium was replaced by freshly, pre-warmed serum-reduced (1% FBS) medium added with 0,1% BSA, in order to reduce particles agglomeration in the culture medium. Particles were exposed to the cells for 4h, 6h and 24h, subsequently the cell medium was replaced with fresh medium containing WST-1 reagent (1:10 v/v).

For co-culture experiments, after 6h exposure to nZnO, WST-1 reagent was mixed with fresh cell culture medium and added to both apical and basal side of the cells for both submerged and ALI culture condition. After 30 min incubation at 37°C, the absorbance of each sample was assessed by Infinitive M200PRO (TECAN, Germany) reader at 450 nm.

The relative viability [%] related to the control samples (untreated cells) was calculated by:

Cell viability = $(OD_{sample}/OD_{control})x100$.

The data on cell viability were reported as relative decrease compared to the control, considered as 100% of viable cells.

2.7 Conventional light microscopy

After exposure to nZnO (5 µg/ml) for 6h, cells inserts were observed under a Zeiss Axioplan light microscope in order to see morphological changes after the treatment.

2.8 RNA isolation

After 6h incubation RNA was isolated form the cells by using RNeasy Mini Kit (Qiagen Hilden, Germany), according to manufacturers' instructions. Briefly, cell culture medium was removed and cells were washed two times with PBS. Cells were lysed with 350 µl of RTL lysis buffer containing 1% (v/v)2-mercaptoethanol. The cell lysate was collected in tube and vortexed and 350 µl of cold ethanol (70%) were added to each cell lysate to precipitate the RNA. 700 µl of lysate was placed onto a Qiagen column and centrifuged at 15000xg for 1min, after that the flow-through was discarded and DNase digestion carried out using DNase I (Qiagen, Hilden, Germany). 350 µl of RW1 were added to the column, and the column was centrifuged at 15000xg 1 min. The flow-through was discarded, the column was placed in a new collection tube and in the same way 500 µl of RPE buffer containing ethanol was added twice and centrifuged first at 15000xg for 1 min and a second time at 15000xg for 2 min. The column was placed in a new collection tube and RNA was eluted from it with 30 µl of RNA-free water by centrifugation at 15000xg for 1 min. RNA was stored at -80°C util use. The amount of RNA was quantified using a ND-1000

spectrophotometer (NanoDrop Technologies) at absorbance ratios of 260 and 280 nm.

2.9 qRT-PCR analysis of pro-inflammatory and oxidative stress markers

Gene level expression of *interleukin-1b* (*Il1b*), *tumor necrosis factor* (*Tnf*) and *heme oxygenase-1* (*Hmox1*) were measured with qRT-PCR. cDNA synthesis was performed using RT First Strand Kit (Life Technologies, Germany) following manufacturers' instructions. For PCR amplification, the above-mentioned cDNA served as template and 3 μL was added together with the specific 5' and 3' primers to the Absolute QPCR SYBR Green Mixes from ABgene (Thermo Fisher Scientific, Germany). Quantitative PCR was performed in a *Taq*Man instrument (*Taq*Man ABI Prism 7700 Sequence Detector System; Perkin- Elmer, Germany) offering the advantage of fast and real-time measurement of fluorescent signals during amplification. The housekeeping gene HPRT (hypoxantine guanine phosphoribosyl transferase) was used as internal reference to normalize the RNA levels of the genes under study. The relative mRNA levels of the target gene was calculated as = $2^{-\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{GAPDH}$.

The following primers were used (sense; antisense):

Tnf: (CACCACGCCTTCTGTCT; GGCTACAGGCTTGTCACTC)

Il1b: (CAACCAACAAGTGATATTCTCCATG;
GATCCACACTCTCCAGCTGCA)

Hmox1: (TGCTCGAATGAACACTCTGG;
TCCTCTGTCAGCATCACCTG)

2.10 Cytokines release

The release of TNF-α protein in the apical and basal compartment of the co-culture in submerged condition was assessed performing the DuoSet ELISA kit (R&D System, Germany), following manufacturers' instructions.

2.11 Statistical analysis

All the experiments was performed in independent triplicates, and data are reported as mean ± SE if not otherwise specified. Statistical analyses were performed with SigmaStat 3.1 software with ANOVA test. Statistical differences were considered to be significant at the 95% level ($p < 0.05$).

3. Results

3.1. Cell viability of monocultures

Cell viability was screened after 4h, 6h and 24h of exposure of LA-4 and MH-S cells to nZnO increasing doses (5-50 µg/ml). After exposure to nZnO, a time- and dose-dependent significant decrease in cell viability was observed in both LA-4 (Fig.3A) and MH-S (Fig.3B) cell lines, evidenced by the viability test WST-1 assay. MH-S cells revealed more sensitive (viability 60%) than LA-4 cells (80%) at 5µg/ml low doses and 6h exposure time. The concentration of 5 µg/ml was used for further co-cultures experiments as it is important to ensure a lower toxicity to cells.

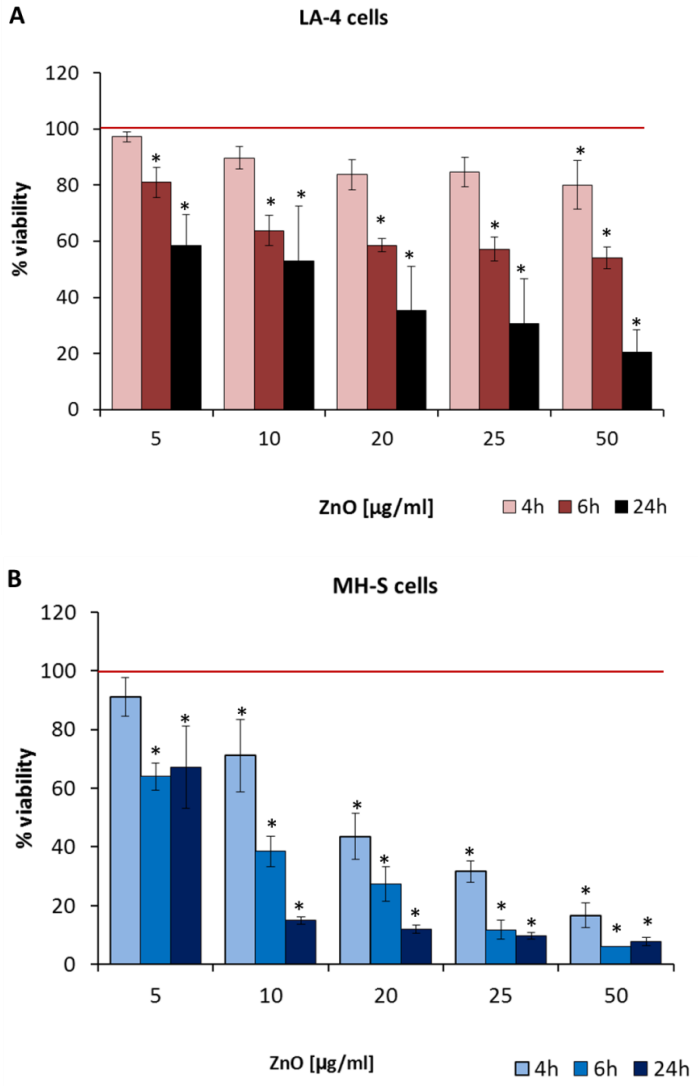


Fig. 3. Cell viability assay (WST-1 test) in monocultures of LA-4 cells (A) and MH-S cells (B) exposed to increasing doses of nZnO for 4, 6 and 24h. The data on cell viability was reported as relative decrease compered to control (represented by the red line), considered as 100% of viable cells.Results are presentated as mean ± SE of at least three independent experiments. *Statistically different from the control ($p<0,05$).

3.2 Cell viability of the co-cultures

The cytotoxicity of nZnO was evaluated by WST-1 test in both submerged and ALI condition. The apical side (AMs) of the co-culture was treated with 5 µg/ml nZnO for 6h and then viability was tested on both compartments. In submerged conditions a slight decrease in cell viability at the apical side of the co-culture system (AMs) was observed. In ALI conditions the viability resulted significantly decreased in both apical (50% of reduction) and basal (40%) compartments (Fig. 4).

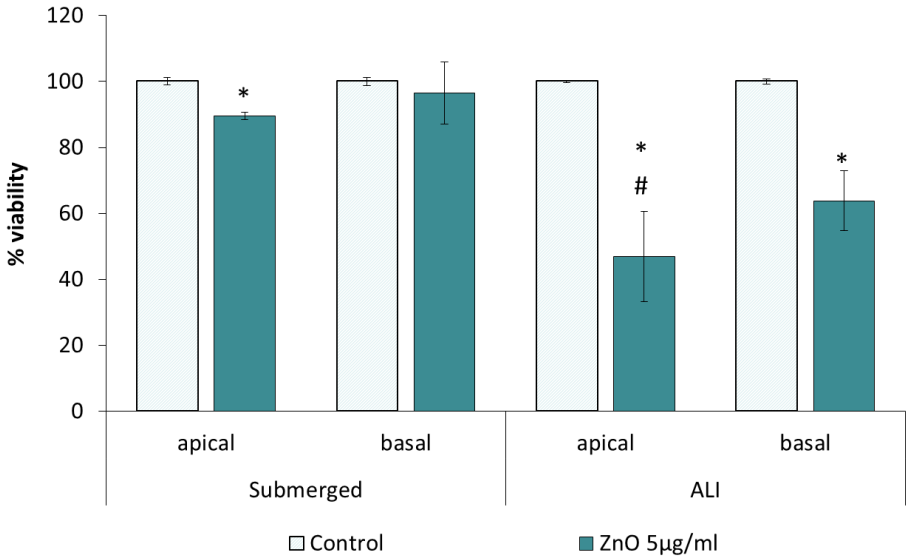


Fig. 4.Cell viability assay (WST-1 test) in co-cultures exposed to 5 µg/ml nZnO for 6h in Submerged and ALI conditions. AECs (LA-4 cells) are present in the apical compartment of the systems, while AMs (MH-S cells) are present in the basal side. Data are presented as mean ± SE of three independent experiments. *Statistically different from the control; #Statistically different respect to the submerged conditions at the same value ($p<0,05$).

Viability data were further confirmed by light microscopy observations. Fig. 5 shows that in submerged condition no changes in cells morphology were visible, while in ALI conditions after exposure to nZnO, AMs (apical) were rounded and shrunk. Furthermore less cells were visible respect to the control, even in the basal compartment (AECs).

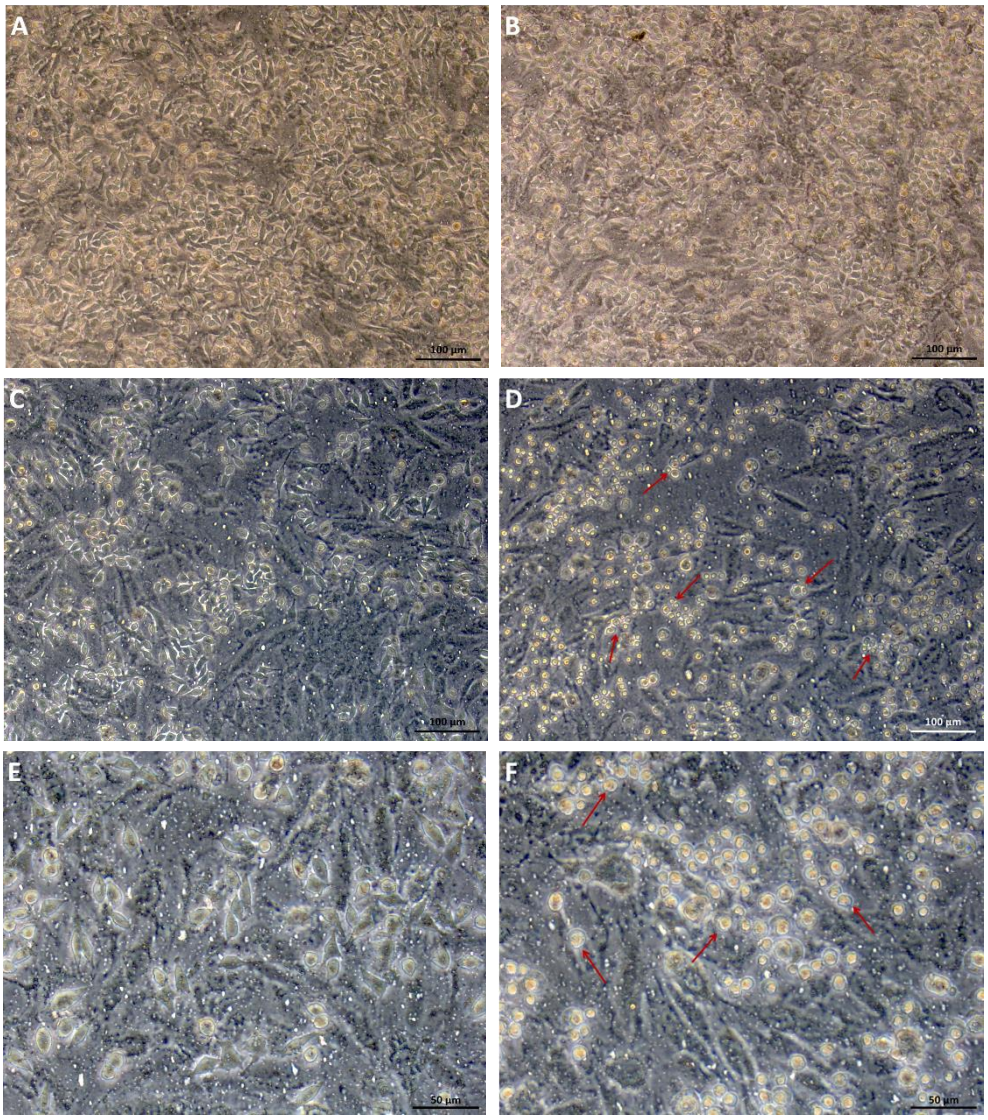


Fig. 5. Light microscopy of AMs and AECs co-cultures Control cells (A, C, E) and cells exposed to 5 µg/ml nZnO (B, D, F) for 6h in submerged (A, B) and ALI conditions (C-F). In submerged condition after the treatment (B) no changes in cells morphology were visible respect to the control (A). Cells exposed to nZnO at the ALI (D, F) have a different morphology respect control cells (C-E), the cells of the apical side (AMs) are rounded (red arrows) and even cells from the basal compartment (AECs) are less visible respect to control samples. Scale bars: A-D= 100µm; E-F= 50 µm.

3.3 Expression of oxidative stress and inflammatory genes

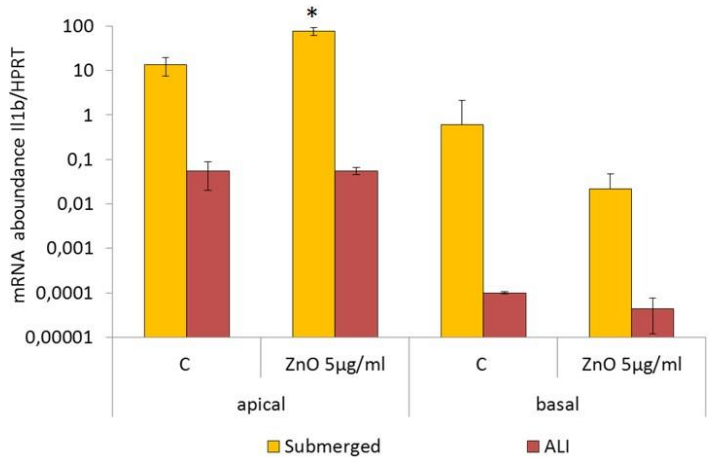
After assessing cytotoxicity of nZnO the effects of NPs on the expression of genes involved in oxidative stress and inflammation has been investigated, as these are two possible mechanisms of action induced by toxicity (Roy et al., 2014b). The mRNA values were normalized to the housekeeping gene HPRT (hypoxantine guanine phosphoribosyl transferase) level.

We investigated *interleukin-1b* (*Il1b*) and *tumor necrosis factor* (*Tnf*) genes, inflammatory genes which mediate the development of numerous lung diseases and are inflammatory markers mainly involved in AMs and AECs after exposure to particles.

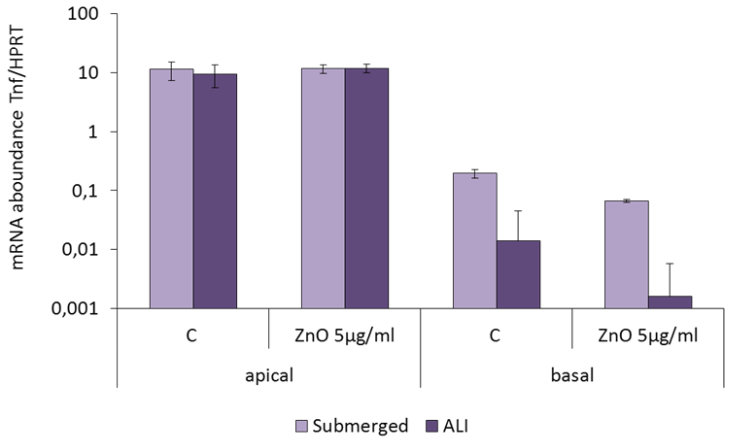
Il1b is a mediator of the inflammatory response. The expression of this gene was lower in samples exposed to the ALI (Fig. 6A). In submerged conditions we observed an increase (6 fold; $p<0,05$) in the apical side of the co-culture system after the treatment, while we didn't observed any effects on *Il1b* expression in ALI conditions. We also observed that *Il1b* is more expressed in macrophages (21 fold) compared to the alveolar epithelial cells. According to these data, nZnO induce inflammation in submerged conditions and the response seems to be mediated by alveolar macrophages that are at the apical compartment of the system. Another marker of inflammation is *Tnf*, a pro-inflammatory cytokine implicated in a variety of diseases, including auto-immune diseases and cancer (Wong et al., 2008). After nZnO exposure there is no effect on *Tnf* expression at the apical side, while a slight but not significant decrease of *Tnf* level in the basal side in submerged conditions (Fig. 6B) was evident.

We investigated also genes related to oxidative stress since inflammation can be a consequence of ROS production (Xia et al., 2008). *Heme*

oxygenase-1 (*Hmox1*) is an important marker of oxidative stress (Li et al., 2002) and in ALI we observed an increase of *Hmox1* mRNA expression in both apical and basal side of the co-cultures. Furthermore, nZnO exposure induced *Hmox1* upregulation 23 fold in lung epithelial cells (basal) compared to only 7 fold in alveolar macrophages (apical) respect to their control (Fig. 6C).



A



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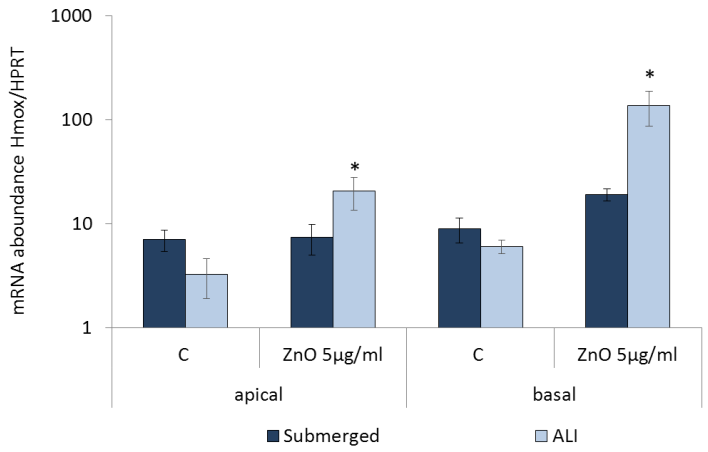


C

Fig. 6. Comparison of the effect of nZnO on proinflammatory (A, B) and oxidative stress marker (C) after exposure of co-culture to ALI and under submerged condition. mRNA expression of proinflammatory cytokines (*Il1b* and *Tnf*) and oxidative stress marker (*Hmox1*) was measured with RT-PCR after 6h of exposure to 5 µg/ml nZnO. The data show mRNA abundance and are presents as geometric means and geometric standard errors of the mean based on at least three independent experiments. The mRNA values were normalized to the housekeeping gene HPRT (hypoxantine guanine phosphoribosyl transferase) level. *Significantly different from the respective control ($p<0,05$)

3.4 *Release of inflammatory cytokines*

The release of the pro-inflammatory cytokine TNF-α in the culture medium of co-cultures in submerged conditions was also investigated. No significant release of TNF-α protein was observed in both compartments (Fig. 7). We have also investigated the release of IL-1β protein in the media of submerged co-cultures but no measurable differences between control and treated samples were observed (data not shown).

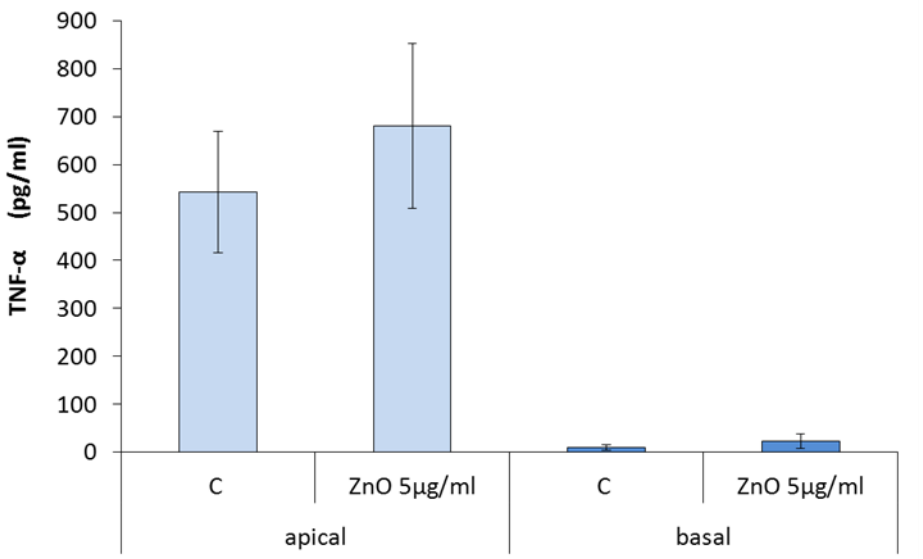


Fig. 7. TNF-α release in the apical and basal medium of co-cultures in submerged conditions. At the dose of 5 µg/ml of nZnO the release of TNF-α is not affected in both apical and basal compartments .Data are expressed as means ± SE of three different independent experiments.

4. Discussion

In many *in vitro* models of the respiratory barrier that have been developed to study cell-particles interactions, cells are immersed in medium and particles suspensions are added in it. This is a non physiological condition because alveolar cells grow at the interface with air (Blank et al., 2006). Due to the well-known criticisms about submerged cell exposure system for assessing inhaled nanoparticles toxicity and effects (Limbach et al., 2005; Paur et al., 2011; Klein et al., 2011; Steinritz et al., 2013), several *in vitro* approaches to ensure exposure systems at the ALI to mimic more realistically the exposure conditions during particle inhalation have been

developed (Bitterle et al., 2006; Savi et al., 2008; Lenz et al., 2009). Further advantages of ALI exposures include the preservation of the physicochemical characteristics of the airborne particles, avoiding particle agglomeration, although the fate of NPs in the lungs, whose surfaces are covered by a thin film of mucus or surfactant, is not completely clear. In addition, particle-medium interactions such as partial dissolution of nZnO in cell culture medium, could be avoided at the ALI (Reed et al., 2012, Xia et al., 2008). The Zn^{2+}/ZnO ratio may be higher in submerged condition due to the dissolution of nZnO in the cell culture medium and this could influence the cellular response (Lenz et al., 2013), enhancing the production of ROS or the expression of oxidative stress markers. Finally, with ALI exposure it is simpler to determine the cell-delivered particle dose than in submerged conditions (Hinderliter et al., 2010), thank also to the use of a quartz microbalance that measures the delivered NPs (Lenz et al., 2009).

Although ALI exposures have become more widely used, very little is known about the differences that ALI and submerged exposure can produce on cellular response.

In this study we developed a co-culture model of the alveolar space using both AMs and AECs and the co-cultures were exposed to nZnO in both submerged and ALI conditions. Viability test and gene expression analysis have been used to screen nZnO toxicity and effects on representative markers of different acute response pathways related to oxidative stress and inflammation.

Several approaches have been used to develop exposure systems at the ALI and in the past they mostly have relied on inertial impaction as deposition mechanism (Fiegel et al, 2003; Bur et al., 2008). The use of impactors or

impingers may impair cell viability because of the high flow rates for effective particles deposition (Mülhopt et al., 2007).

In the present study we used the Air–Liquid Interface (ALI) Cell Exposure–Cloud system (ALICE-CLOUD) proposed by Lenz et al. (2009), a more gentle technique of particles deposition. In this system NPs are aerosolized by a nebulizer and uniformly deposited via cloud formation and particles settling onto cells.

Our data show that after nZnO exposure there was a difference in cell viability comparing submerged and ALI cultivations. nZnO exposure at the ALI resulted more toxic for cells, especially we observed a reduction in cell viability in both compartments of a co-culture composed of AMs in the apical side (50% reduction of cell viability) and AECs in the basal side (40% reduction of cell viability) of a Transwell insert. These data are in contrast with previous results from Lenz and colleagues (2013). According to these authors, cell viability of human alveolar cells (A549) was similar after ALI or submerged exposure to nZnO, here instead we report a significant difference cells viability comparing submerged and ALI exposure conditions. The possible explanation of this difference may be related to a specific type sensitivity to nZnO, here we used murine alveolar cells (LA-4) instead of A549 cell line. Furthermore LA-4 were co-cultured with MH-S (AMs) for a longer time of exposure (6h instead of 3h). The presence of AMs that interact with particles can modulate the response to NPs due to the release of proinflammatory mediators (Hiraiwa and van Eeden 2013) also in alveolar cells. An preferable *in vitro* model representative of the cross-talk between AMs and AECs would be a co-culture in which both AMs and AECs are seeded in the same compartment

of a Transwell insert. We have tried to develop such model, but even if it is suitable for submerged exposure, we observed a loss of viability in ALI conditions at early time exposure (2h) even in control cells (Supplementary data - CHAPTER VI, Fig. S1 and S2). Since the aim of our work was to investigate the difference between ALI and submerged exposure condition to nZnO, we decided to abandon this model.

We hypothesized that the reason of the mortality of the cells may be due to the incapacity of LA-4 to secrete surfactant, which is very important for cells survival. Another problem may be that the culture medium is not able to reach the AMs that are seeded on the confluent layer of AECs cells, leading to death.

All together this data suggest that ALI system of exposure can influence cell viability and subsequent biological responses; such system requires therefore preliminary phases of set up to avoid misinterpretation of results. The greater availability of oxygen at ALI could lead to an unintentional ROS production (Kameyama et al., 2003), and the effects caused by particles could be masked.

The responses of the co-culture with AMs in the apical side and AECs in the basal one were analysed considering markers of different acute response pathways related to oxidative stress and inflammation. There are many evidences that the *heme oxygenase-1* (*Hmox1*) gene is upregulated by air pollution ultrafine particles in human cells (Li et al., 2002; Becker et al., 2005; Akhater et al., 2014). In the lung this protein has antioxidant and cytoprotective effects (Choi et al., 1996) and is a sensitive marker for oxidative stress (Li et al., 2002).

Our data show that in ALI condition the expression of the *Hmox1* gene is upregulated for both apical and basal compartment after nZnO exposure. In addition, nZnO exposure induced *Hmox1* expression higher in lung epithelial cells (23 fold) compared to alveolar macrophages (only 7 fold). These data suggest that the direct effect of nZnO on AMs may determine a secondary effect on AECs through the release of inflammatory cytokines or chemical mediators (e.g. ROS). Moreover, nZnO could also cross the Transwell and have on AECs a direct effect that might influence the cellular response. Future investigations will be addressed in order to verify these hypotheses.

As inflammatory genes we investigated *interleukin-1b* (*Il1b*) and *tumor necrosis factor* (*Tnf*) because they are crucial mediators in the development of numerous inflammatory lung diseases (Chung et al., 2008) and they are the first mediators that occur in the inflammatory response after particles exposure (Hetland et al., 2005; Hiraiwa and van Eeden 2014). nZnO have induced *Il1b* expression in submerged condition, indicating that in this co-culture system AMs are the cells that trigger the inflammatory response.

These results from *Hmox1* and *Il1b* genes expression after nZnO exposure are in accordance with previous work of Lenz and coworkers (2009), in which the authors have observed in A549 cells exposed to nZnO an increase of *Hmox1* mRNA expression at the ALI and of another pro-inflammatory cytokine gene, IL-8, in submerged conditions.

These data suggest that submerged conditions are preferable for the investigation of the potential inflammatory status induced by NPs. Nevertheless, we have not observed any release of TNF-α protein in submerged condition, in accordance with the result from mRNA expression.

The expression of *Tnf* after nZnO exposure is controversial. According to the majority of *in vivo* and *in vitro* studies nZnO are able to induce *Tnf* expression (Chang et al., 2013; Hanley et al., 2009), but in other studies conducted in immortalized rodent lung epithelial cells, alveolar macrophage cell lines and primary alveolar macrophages, nZnO fail to induce TNF-α protein (Sayes et al., 2007; Beyerle et al 2009). The ability of nZnO to induce or not induce high levels of TNF-α may be due to the different cell types and to time of exposure to particles selected in the experimental plan (Hanley et al., 2009).

We have also investigated the release of IL-1β protein in the media of submerged co-cultures but no differences between control and treated samples were noticed (data not shown). The lack in IL-1β secretion may be due to the mechanisms of secretion of this cytokines. The release of the soluble pro-inflammatory cytokines IL-1β occurs after the cleavage of the cytoplasmic pro-interleukin IL-1β in its active form IL-1β by a molecular machinery called inflammasome (Yu and Finlay 2008). Among the different inflammasomes, the NLRP3 is the most characterized. The activation of this complex has been related to the exposure of different pathogens-associated molecular patterns (PAMPs) and host-derived molecules (Schroder et al., 2010) as well as PM (Bengalli et al., 2013) and NPs (Yadzi et al., 2010). Although some NPs (TiO_2 and SiO_2) are able to activate this complex, nZnO exposure has been reported to fail to induce the expression of NLRP3 and the subsequent secretion of IL-1β (Yadzi et al., 2010; Xia et al., 2013), according to our results.

The presented data have shown significant differences of cellular response after nZnO exposure under submerged and ALI conditions, suggesting that

NPs *in vitro* studies should be done considering the more realistic ALI cultivation and exposure in addition to the well-established submerged conditions.

5. Conclusions

The concern about the method of exposure for assessing NPs effects is highly increased in the last decade (Paur et al., 2011). The evidence that submerged *in vitro* culture have many limitations, especially for the study of inhaled particles, poses the need to develop new methods for reaching a better knowledge about particles effective risk to human health.

In this study the *in vitro* responses of a co-culture of alveolar epithelial cells and alveolar macrophages to nZnO was compared in ALI exposure conditions and submerged ones. Cells were cultured on opposite side of a Transwell insert giving us the chance to distinguish the different NPs-induced responses from the two different cell lines (apical AMs and basal AECs) in co-culture.

The results have evidenced an increased cell mortality in co-cultures exposed at the ALI and a pronounced stress response, characterized by the oxidative marker expression (*Hmox1*), compared to results from submerged conditions. Nevertheless, in the submerged exposure system we also observed a significant induction in AMs cells exposed to nZnO of inflammatory genes. These results support the idea that the way of NPs administration is pivotal for the analysis of NPs-induced biological effects.

Furthermore, the crosstalk that occurs between different cell types at the alveolar space should be taken into account in order to understand which cells trigger certain responses. *In vitro* alveolar barrier models are useful

tools to understand the mechanisms of NPs-induced effects and they can also be relevant for the study of drug delivering to lungs. The use of an ALI exposure system could be preferable, because it represents a more realistic scenario. However, quantitative comparisons of the cellular response under submerged and ALI condition are still very limited and they need implementation in order to have a better knowledge of the indirect effects of the exposure system on cells. Comparative studies of different exposure systems, with well characterized NPs, will be urgently needed in the future for the correct evaluation of the potential adverse health effects of inhaled NPs.

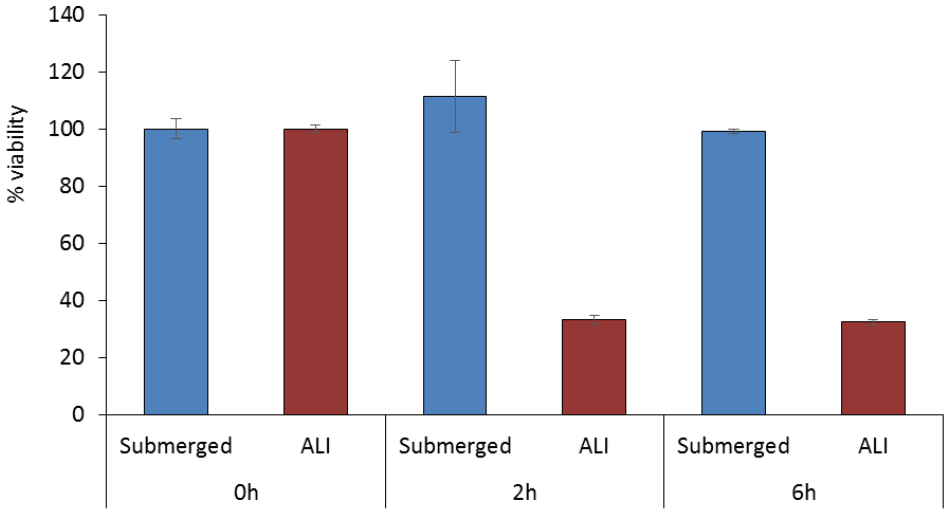
Supplementary data

Co-cultures set-up on Transwell inserts

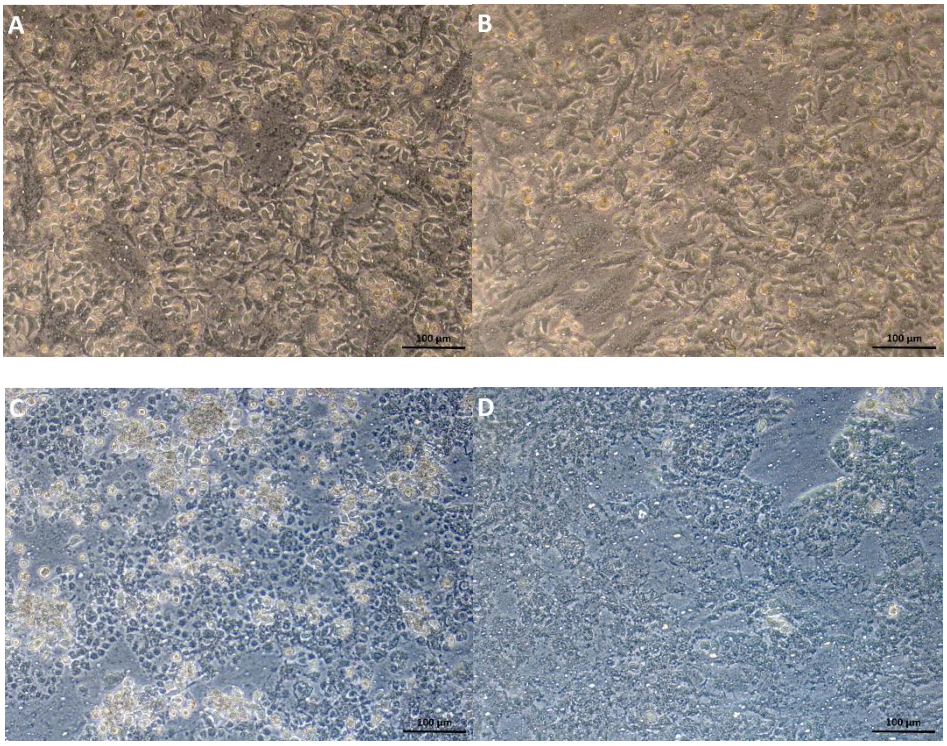
LA-4 were seeded at the density of $0{,}5x10^6$ cells/insert on a Transwell insert (BD Falcon, transparent PET membrane, effective growth area 4,2 cm^2, 0.4µm pore size, $1.6x10^6$ pores/cm^2) and let them adhere for 24 h in the incubator at 37°C, 5% CO_2. After the cells had formed a confluent monolayer (24h), MH-S cells (in a ratio of 1:4 meaning $0{,}125x10^6$ cells/inserts) were seeded on the top of the epithelial cells and incubated for 24h in submerged conditions. Afterwards co-cultures were cultivated in medium for submerged experiment for 2h and 6h, while for ALI experiment medium culture were removed, cells were exposed at the ALI to a suspension of medium and then incubated for 2 and 6h with fresh medium only in the basal side of the system.

Supplementary results

In Figure S1 the time course of cell viability results of the co-cultures exposed in ALI and submerged conditions is presented. In this model cells are seeded in the same compartment (apical side) of a Transwell insert. We observed that in submerged conditions cells maintained the same viability value during time (2h and 6h). In ALI conditions, on the contrary, the viability resulted decreased (70% of reduction respect to time 0) also after 2h of exposure at the ALI. Data were also confirmed by light microscopy analysis (Fig. S2).



Supplementary Fig. S1. Time course of cell viability assay (WST-1 test) of co-cultures exposed in submerged (blue bars) and ALI conditions (red bars) for 2h and 6h without apical addition of nZnO. Data are presented as mean ± SD of three independent experiments.



Supplementary Fig S2. Light microscopy of LA-4 and MHS co-cultured on the same compartment of a Transwell insert (pores diameter 0,4 µm). Cells were cultivated in submerged (A,B) and ALI (C,D) conditions for 2 and 6h. Control co-cultures (no nZnO exposure) were exposed in submerged (A,B) and ALI (C,D) conditions for 2h (A, C) and 6h (B, D). After 2h of exposure to the ALI AMs are rounded and detached from the AECs. AECs have shrunk shape if compared to cells in submerged condition. After 6h at the ALI (D) no AMs were visible. Scale bars= 100µm.

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CHAPTER VII

GENERAL CONCLUSIONS

The production at industrial scale of manufactured NPs increases the unintentional occupational and environmental human exposure to these new materials. Among them, MONs are widely used in several fields of application, including daily good-lifeproducts. These circumstances increase the concern about NPs hazard to exposed persons. Researchers are called to fulfil the knowledge regarding NPs adverse effects on humans and environment in order to develop safer nanomaterials and to define good requisites for a stricter regulation about NPs by the European Commission (Foss and Baun 2012). Furthermore the directive on the protection of animals used for scientific purposes (Directive 2010/63/EU revising Directive 86/609/EEC) establishes the need to test new chemicals by using, whenever it is possible, *in vitro* alternative methods.

In this perspective the present work improves the knowledge on the toxicological effects of a largely used MONs, nZnO, by using different *in vitro* models representative of the respiratory barrier. Thank to this approach it was possible to evaluate several NPs-induced biological endpoints.

nZnO cytotoxicity on the epithelial barrier was screened in *in vitro* monocultures of epithelial alveolar cells (NCI-H441 cell line). The cytotoxic effects were related to ROS formation, thus confirming the overall accepted paradigm on the importance of oxidative stress in NPs-related biological effects. Furthermore ROS formation was linked to the increased release of intracellular free Zn^{2+} induced by nZnO. Although further investigations are

needed to confirm this hypothesis, the increased levels of intracellular Zn^{2+} was partially due to the uptake of extracellular Zn^{2+} released by nZnO, but it might also depend on an intracellular dissolution of NPs and consequent release of Zn^{2+} ions. Future researches will be focused to confirm these hypotheses through the analysis of the amount of Zn^{2+} released from nZnO and through the localization of Zn^{2+} in the cellular compartment, focusing primarily on lysosomes.

However the ability of cells to activate mechanisms of protection was also showed. Cell exposed to nZnO activated metallotioneins (MT) to scavenge free zinc ions. Taken together these results demonstrate that nZnO cytotoxic effects, mediated by radical species, are the results of the impairment of mechanisms of defense activated after exposure.

Importantly at a high dose (50 µg/ml) and at early time of exposure (6 h) the toxic effects of nZnO determined also the disruption of the junctions, which are essential for an integral alveolar barrier. In exposed monocultures nZnO was able to alter cellular morphology as well as the formation of TJs among cells, as demonstrated by the reduced expression of the junctional protein ZO-1.

The need to reduce and replace *in vivo* models requires the development of innovative *in vitro* systems able to mimic the important interplays that happen in tissues. Here an innovative air-blood barrier (ABB) has been developed and used to assess nZnO *in vitro* toxicity. The co-culture system composed of human epithelial alveolar cells (NCI-H441) and endothelial cells of the micropulmonary circulation (HPMEC-ST1.6R cell line) was used to evaluate the importance of the cross-talk among these cells for the release of pro-inflammatory mediators (IL-6 and IL-8) and soluble adhesion

molecules (sICAM-1 and sVCAM-1), which are pivotal markers of endothelial inflammation and dysfunction.

Although the co-culture models permit more relevant *in vitro* studies, some issues need still to be addressed. For example the limited amount of cells available on the Transwell inserts reduces the biological endpoint which can be analyzed in each test. ROS and other fluorescent assays are moreover limited by the presence of the two cell types attached to the insert.

However the integrity of the barrier, in term of trans epithelial electric resistance (TEER), was evaluated. This biological endpoint is of primary interest to understand if NPs are in general able to disrupt the functional homeostasis of biological barrier. Here we reported that nZnO at the used doses determined only non significant effects on the barrier functionality. Compared to the results obtained in the monoculture, these data suggest that the interplay among different cell types is important also for the maintenance of the ABB. Finally, significant results were obtained on the ability of nZnO to induce endothelium activation after exposure of epithelial cells. The increased release of IL-6 and sVCAM-1 were detected in the basal compartment (endothelial cells) of the system, suggesting that endothelial inflammation and vascular damage occurred, even if NPs are not in direct contact with endothelial cells.

These data demonstrate that the ABB model here reported is a suitable system for studying the cross-talk among the different cell types that are present at the respiratory barrier, evidencing that different mediators are involved in the local (pulmonary, IL-8) and systemic (endothelial, IL-6 and sVCAM-1) inflammatory events.

The system was further implemented with a monocytic cell line (human THP-1 cells) added in the basal compartment in order to investigate the role of immunomodulation in the response to NPs. The results confirmed the importance of monocytes on inflammatory responses, evidenced by an increased release of IL-8 from both apical and basal compartment of the tri-culture and a basal increase of sICAM-1. The differences with the previous co-culture system on interleukins and sVCAM-1 release furthermore suggest that the presence of monocytes may influence also the endothelial response.

These data evidence that, by increasing the complexity of the model, more realistic and informative data may be obtained although the explanation of the results requires further investigations. The cross-talk among several different cells influences the ABB inflammatory responses to NPs exposure and these results may drive future research for the development of safer nanomaterials.

Future studies should be focused on the investigation of other mediators, such as the release from endothelial cells of E-selectin and nitric oxide (NO), which are more specific markers of endothelial activation and cardiovascular associated diseases (Kjærgaard et al., 2013; Davignon et al; 2004). Furthermore, the adhesion of monocytes to endothelial cells will be also another interesting goal to assess, because a possible correlation between NPs exposure and an enhanced adhesion between these cell types will be an important step forward for the understanding of NPs-induced vascular effects, such as the potential onset of atherosclerotic plaques.

The response to NPs can be also influenced by the way of administration and in this perspective another co-culture system was developed using murine alveolar macrophages (AMs) and alveolar epithelial cells (AECs). This model was exposed to nZnO by using two different exposure methods: submerged and air-liquid interface (ALI) systems. Data have evidenced an increased cell mortality in co-cultures exposed at the ALI and a pronounced stress response, characterized by the oxidative marker expression of specific genes (*Hmox*), after nZnO exposure in ALI conditions, but not in submerged ones, in both AMs and AECs present in the co-culture. Furthermore, AECs seems to be more sensitive to the treatment since the expression of *Hmox* was higher in the AECs respect to AMs.

Nevertheless, in the submerged exposure system nZnO induced an increased gene expression of the pro-inflammatory gene *Il1b* in AMs, suggesting that these cells trigger the inflammatory response. These results support the idea that the way of NPs administration is crucial for assessing specific endpoints. Furthermore, the separation in two distinct compartments of the co-culture system was essential to understand the cross-talk that occurs between different cell types at the alveolar barrier and to delineate the importance of each cell type in triggering different biological responses.

The discussion is therefore still open on the more realistic and preferable way of exposure of *in vitro* systems to NPs. ALI exposure systems seem preferable because they represent a more realistic scenario, however the quantitative comparison of the cellular response under submerged and ALI condition are still very limited to select a gold-standard method. The requirement of a reference protocol of exposure is mandatory for improving our knowledge on the potential adverse effects of inhaled particles.

In conclusion the proposed *in vitro* models of the respiratory epithelium, in particular co- and tri-culture systems, give a more realistic view of the mechanisms involved in NPs-induced responses. In particular with these systems we clearly evidenced the importance of cellular cross-talk for the induction by NPs of endothelial inflammation and damage, which are two important biological endpoints that occur also *in vivo* after NPs inhalation. However it is important to consider that the environmental concentrations of nZnO are lower than the doses used in this study and that the currently recommended Occupational Safety and Health Administration (OSHA) standard for ZnO fume corresponds to a daily alveolar surface dose of 1.1-5.4 ng/cm^2 (Lenz et al., 2013). In spite of our unrealistic doses of exposure, the *in vitro* models here presented are useful tools for toxicological analysis of substances and studies of cellular response mechanisms, even if lower cellular doses may be desirable. Moreover, although *in vitro* test systems which mimic the *in vivo* microenvironment may not completely replace *in vivo* studies, the implementation of these models as well as of the ALI exposure system, can provide important information on relevant biological effects after inhalational of nanomaterial. The future research shall bring together the benefits from reproducible *in vitro* models of the ABB, for evaluation of nanotechnology safety.

LIST OF ABBREVIATIONS

ABB	Air-Blood Barrier
AECs	Alveolar Epithelial Cells
AEICs	Alveolar Epithelial Type I Cells
AEIICs	Alveolar Epithelial Type II Cells
Ag-NP	Silver Nanoparticles
ALI	Air-Liquid Interface
AMs	Alveolar Macrophages
BSA	Bovine Serum Albumin
CAMs	Cellular Adhesion Molecules
CuO	Copper Oxide
DCs	Dendritic cells
DLS	Dynamic Light Scattering
ECs	Endothelial Cells
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
GSH	Glutathione S-hydrogenease
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
Hmox1	Heme-oxygenase-1
ICAM-1	Intercellular Adhesion Molecule-1

ICP-MS	Induced Coupled Plasm Mass Spectrometry
Il1b	Interleukin-1b
IL-1β	Interleukin-1β protein
IL-6	Interleukin-6
IL-8	Interleukin-8
MONs	Metal oxide nanoparticles
MT	Metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMs	Nanomaterials
NPs	Nanoparticles
nZnO	Zinc Oxide Nanoparticles
PBS	Phosphate Buffered Saline
PM	Particulate Matter
PMA	Phorbol-12-Myristate-13-Acetate
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
sCAMs	Soluble Cellular Adhesion Molecules
SEM	Scanning Electron Microscopy
sICAM-1	Soluble Intercellular Adhesion Molecule-1
SiO2	Silica Dioxide

SOD	Superoxide Dismutase
sVCAM-1	Soluble Vascular Cellular Adhesion Molecule-1
TEER	Trans-Epithelial Electrical Resistance
TEM	Transmission Electron Microscopy
TiO_2	Titanium Dioxide
TJs	Tight Juntions
Tnf	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor-α protein
UFP	Ultrafine Particles
VCAM-1	Vascular Cellular Adhesion Molecule-1
ZO-1	Zonula Occludens-1

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